Methods for identifying proteins with starch phosphorylating enzymatic activity

Description

The present invention relates to a method for identifying proteins involved in the phosphorylation of starch, and nucleic acids which code for such proteins. The invention further relates to plant cells and plants which exhibit an elevated activity of a protein identifiable using the method according to the invention. Plant cells and plants of this type synthesise a modified starch. The present invention therefore also relates to the starch synthesised by the plant cells and plants according to the invention as well as to methods for the manufacture of this starch and to the manufacture of starch derivatives of this modified starch.

With regard to the increasing importance currently attributed to plant constituents as renewable raw material sources, one of the tasks of biotechnological research is to endeavour to adapt these plant raw materials to suit the requirements of the processing industry. Furthermore, in order to enable regenerating raw materials to be used in as many areas of application as possible, it is necessary to achieve a large variety of materials.

Polysaccharide starch is made up of chemically uniform base components, glucose molecules, but constitutes a complex mixture of different molecule forms, which exhibit differences with regard to the degree of polymerisation and branching, and therefore differ strongly from one another in their physical-chemical characteristics.

Discrimination is made between amylose starch, an essentially unbranched polymer made from alpha-1,4-glycosidically linked glucose units, and amylopectin starch, a branched polymer, in which the branches come about by the occurrence of additional alpha-1,6-glycosidic links. A further essential difference between amylose and amylopectin lies in the molecular weight. While amylose, depending on the origin of the starch, has a molecular weight of $5x10^5$ - 10^6 Da, that of the amylopectin lies between 10^7 and 10^8 Da. The two macromolecules can be differentiated by their molecular weight and their different physical-chemical characteristics, which can most easily be made visible by their different iodine bonding characteristics.

Amylose has long been regarded as a linear polymer, consisting of alpha-1,4-glycosidically linked alpha-D-glucose monomers. In more recent studies, however, the presence of alpha-1,6-glycosidic branching points (ca. 0.1%) has been shown (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92).

The functional properties such as, for example, the solubility, retrogradation behaviour, water binding capacity, film-forming properties, viscosity, gelatinisation properties, freeze-thaw stability, acid stability, gel strength and the starch granule size of starches are influenced among other things by the amylose/amylopectin ratio, molecular weight, side-chain distribution pattern, ion content, lipid and protein content, average starch granule size, starch granule morphology etc. The functional properties of starch are also influenced by the phosphate content, a non-carbon component of starch. Here, differentiation is made between phosphate, which is bound covalently in the form of monoesters to the glucose molecules of the starch (described in the following as starch phosphate), and phosphate in the form of phospholipids associated with the starch. In addition to the phosphate content, the influence on the functional properties of the starch is in this case also dependent on the form (starch phosphate or phospholipid) in which the phosphate occurs in the starch (Jane et al., 1996, Cereal Foods World 41 (11), 827-832).

The starch phosphate content varies according to the species of plant. Therefore, certain maize mutants, for example, synthesise a starch with increased starch phosphate content (waxy maize 0.002% and high-amylose maize 0.013%), while conventional types of maize only have traces of starch phosphate. Likewise small quantities of starch phosphate are found in wheat (0.001%) whereas no starch phosphate could be detected in oats and sorghum. In rice mutants likewise, less starch phosphate was found (waxy rice 0.003%) than in conventional species of rice (0.013%). Significant quantities of starch phosphate were detected in tuber- or root-storing starch synthesising plants such as, for example, tapioca (0.008%), sweet potato (0.011%), arrowroot (0.021%) or potato (0.89%). The percentage values for the starch phosphate content quoted above refer to the dry weight of starch in each case, and have been determined by Jane et al. (1996, Cereal Foods World 41 (11), 827-832).

Starch phosphate can be present in the form of monoesters at the C-2, C-3 or C-6 position of the polymerised glucose monomers (Takeda and Hizukuri, 1971, Starch/Stärke 23, 267-272). The phosphate distribution of the starch phosphate in starch synthesised by plants is generally distinguished by the fact that about 30% to 40% of the phosphate residues are covalently bound in the C-3 position and about 60% to 70% of the phosphate residue is covalently bound in the C-6 position of the glucose molecule (Blennow et al., 2000, Int. J. of Biological Macromolecules 27, 211-218). Blennow et al. (2000, Carbohydrate Polymers 41, 163-174) have determined a starch phosphate content which is bound in the C-6 position of the glucose molecule for various starches, such as for example, potato starch (between 7.8 and 33.5 nMol per mg of starch, depending on the type), starch from various *Curcuma* species (between 1.8 and 63 nMol per mg), tapioca starch (2.5 nMol per mg of starch), rice starch (1.0 nMol per mg of starch), mung bean starch (3.5 nMol per mg of starch) and sorghum starch (0.9 nMol per mg of starch). These authors have been unable to show any starch phosphate bound at the C-6 position in barley starch and starches from different waxy mutants of maize. Up to now, it has not been possible to establish a connection between the genotype of a plant and the starch phosphate content (Jane et al., 1996, Cereal Foods World 41 (11), 827-832). Thus, at the

present time it is not possible to influence the content of starch phosphate in plants by means of breeding.

In transgenic plants the quantity of starch phosphate in storage starches can be varied. Thus, storage starch from potato plants which exhibit a reduced activity of soluble starch synthase III (Abel et al., 1996, The Plant Journal 10(6), 9891-991), branching enzyme I (BEI) (Safford et al., 1998, Carbohydrate Polymers 35, 155-168), branching enzyme II (BEII) (Jobling et al., 1999, The Plant Journal 18, 163-171), BEI and BEII (Schwall et al., 2000, Nature Biotechnology 18, 551- 554), a disproportionation enzyme (WO 96 27673) or a disproportionation enzyme and a BEI (WO 95 07355), show an elevated content of starch phosphate compared with starch from corresponding wild type plants. However, the alteration in the starch phosphate content in these plants is not due to the proteins whose activity is reduced in these plants, being directly involved in the introduction of phosphate residues into the starch. The increase in the content of starch phosphate in the transgenic plants concerned is thus not a primary but a secondary effect which is brought about by reduction of the corresponding proteins. The reason for the increase in the content of starch phosphate as a result of modification of said protein activities is as yet still unexplained. Thus, it is not possible to specifically modify the content of starch phosphate by modifying protein activities which only influence the starch phosphate content by a secondary effect. Furthermore, modifying the activities of proteins which as a secondary effect have an influence on the content of starch phosphate in plants, at the same time also brings about further modifications in the starch, such as, for example: changes in the amylose/amylopectin ratio and/or the length of the side chains of the amylopectin which consistitutes the primary effect of the changes in such protein activities.

Previously, only one protein has been described, which mediates the introduction of covalent bonds of phosphate residues to the glucose molecules of starch. This protein, frequently designated as R1 in the scientific literature, is bound to the starch granules of the storage starch in potato tubers (Lorberth et al., 1998, Nature

Biotechnology 16, 473-477) and has the enzymatic activity of an alpha-glucan water dikinase (E.C. 02.07.09.4). In the reaction catalysed by R1 the educts alpha-1,4-glucan (starch), adenosinetriphosphate (ATP) and water are converted to the products glucan phosphate (phosphorylated starch), monophosphate and adenosine monophosphate. In this case, the gamma phosphate residue of the ATP is transferred to water and the beta phosphate residue of the ATP is transferred to the glucan (starch). R1 transfers *in vitro* the beta phosphate residue of the ATP to the C-6 and the C-3 position of the glucose molecules of alpha-1,4-glucans. The ratio of C-6 phosphate to C-3 phosphate which is obtained in the *in vitro* reaction corresponds to the ratio which is present in starch isolated from plants (Ritte et al., 2002, PNAS 99, 7166-7171). As about 70% of the starch phosphate present in potato starch is bound to the glucose monomers of the starch in the C-6 position and about 30% in the C-3 position, this means that R1 preferably phosphorylates the C-6 position of the glucose molecules. Furthermore, it has been shown by using amylopection from maize that, amongst other things, R1 can phosphorylate alpha-1,4-glucans which do not yet contain covalently bound phosphate (Ritte et al., 2002, PNAS 99, 7166-7171), i.e., R1 is able to introduce phosphate *de novo* into alpha-1,4-glucans.

The amino acid sequence of R1 contains a domain which exhibits a high degree of homology to known pyruvate phosphate dikinases (PPDK domains) and known pyruvate water dikinases (PPS domains) and contains a histidine residue conserved in PPDK and PPS domains. During the transfer of phosphate residues of the ATP to alpha-1,4-glucans (starch) a phosphorylated R1 protein is formed as intermediate product, with a phosphate residue being present, covalently bound to the histidine residue conserved in the PPDK or the PPS domain (Mikkelsen et al., 2004, Biochemical Journal 377, 525-532).

Nucleic acid sequences and amino acid sequences corresponding to these, coding for an R1 protein, are described from various species, such as for example, potato (WO 97 11188, GenBank Acc.: AY027522, Y09533), wheat (WO 00 77229, US 6,462,256, GenBank Acc.: AAN93923, GenBank Acc.: AR236165), rice (GenBank

Acc.: AAR61445, GenBank Acc.: AR400814), maize (GenBank Acc.: AAR61444, GenBank Acc.: AR400813), soya bean (GenBank Acc.: AAR61446, GenBank Acc.: AR400815), citrus (GenBank Acc.: AY094062) and *Arabidopsis* (GenBank Acc.: AF312027).

Wheat plants which exhibit an elevated activity of an R1 protein as a result of overexpression of an R1 gene from potato are described in WO 02 34923. Compared with the corresponding wild type plants, in which no starch phosphate could be detected, these plants synthesise a starch having significant quantities of starch phosphate in the C-6 position of the glucose molecules.

Further proteins which catalyse a reaction which introduces covalently bound phosphate groups into the starch have not so far been described. Enzymes, which preferably introduce phosphate groups in the C-3 position and/or the C-2 position of the glucose molecules of starch, are also not known. Thus, apart from increasing the content of starch phosphate in plants, there are no available ways for specifically influencing the phosphorylation of starch in plants, modifying the phosphate distribution within the starch synthesised by plants and/or further increasing the content of starch phosphate.

It is thus the object of the present invention to provide methods and means for producing plants synthesising a modified starch having elevated phosphate content and/or modified phosphate distribution. as well as to provide plant cells and/or plants which synthesise such a modified starch.

This problem is solved by the embodiments described in the claims.

Thus, the present invention relates to a method for identifying a protein which has an elevated binding activity towards phosphorylated alpha-1,4 glucans, compared to non-phosphorylated alpha-1,4 glucans, wherein

a) protein extracts in preparations separated from one another are incubated with

i phosphorylated alpha-1,4 glucans and

ii non-phosphorylated alpha-1,4 glucans,

b) proteins specifically bound to the

i phosphorylated alpha-1,4 glucans from step a) i and

ii proteins specifically bound to the non-phosphorylated alpha-1,4 glucans from step a) ii

are dissolved in preparations separate from one another and

c) proteins are identified which exhibit an elevated binding activity towards phosphorylated alpha-1,4 glucans used in step b) i, compared to non-phosphorylated alpha-1,4 glucans used in step b) ii.

In a further embodiment of the method according to the invention for identifying a protein which exhibits an elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4 glucans, the alpha-1,4 glucan to which a higher binding activity exists is a starch, preferably a granular starch.

A further embodiment of the method according to the invention for identifying a protein which exhibits an elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4 glucans, relates to a method for identifying a protein which has a molecular weight derived from the amino acid sequence of 120 kDa to 145 kDa, preferably 120 kDa to 140 kDa, particularly preferably 125 kDa to 140 kDa, especially preferably 130 kDa to 135 kDa.

In a further embodiment, the method according to the invention relates to a method for identifying a protein which exhibits an elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4 glucans, wherein the binding activity to P-alpha-1,4-glucans is increased at least three times, preferably at least four times, particularly preferably at least five times and especially preferably at least six times compared to the binding activity to non-phosphorylated alpha-1,4-glucans.

The quantity of proteins which bind to P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans can, for example, be determined by immunological methods such as Western Blot Analysis, ELISA (Enzyme Linked Immuno Sorbent Assay) or RIA (Radio ImmunoAssay).

Methods for manufacturing antibodies, which react specifically with a certain protein, i.e. which bind specifically to said protein, are known to the person skilled in the art (see, for example, Lottspeich and Zorbas (Eds.), 1998, Bioanalytik, Spektrum akad, Verlag, Heidelberg, Berlin, ISBN 3-8274-0041-4). The manufacture of such antibodies is offered by some companies (e.g. Eurogentec, Belgium) as a contract service. One possible way for manufacturing antibodies which react specifically with a protein according to the invention is described below (see Example 11).

By comparing the dissolved P-alpha-1,4 glucan-binding proteins obtained by implementing the method according to the invention for identifying a protein which has an elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans, with the dissolved non-phosphorylated alpha-1,4-glucan-binding proteins, which are obtained, it is possible to identify proteins which have an elevated binding activity towards P-alpha-1,4 glucans compared to non-phosphorylated alpha-1,4 glucans.

In a further embodiment of the method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-

glucans compared to non-phosphorylated alpha-1,4 glucans, the P-alpha-1,4-glucan protein complexes obtained by incubating protein extracts with P-alpha-1,4-glucans according to step a) i and the non-phosphorylated alpha-1,4-glucan protein complexes obtained by incubating protein extracts with non-phosphorylated alpha-1,4-glucans according to step a) ii are separated from the proteins not bound to the relevant alpha-1,4-glucans. In this case, the separation takes place separately for the respective incubation solutions after process step a) i or after process step a) ii.

In a further embodiment of the method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4 glucans, the proteins dissolved according to step b) i or b) ii are separated from the alpha-1,4 glucans used in the method according to the invention according to step a) i or step a) ii.

In the method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4 glucans, the dissolved proteins obtained according to process step b) i can either comprise a single protein or a plurality of proteins. The proteins dissolved according to process step b) ii can also either comprise a single protein or a plurality of proteins. Should the dissolved P-alpha-1,4-glucan-binding proteins or the dissolved non-phosphorylated alpha-1,4 glucan-binding proteins respectively comprise a plurality of different proteins, these are separated from one another if necessary.

In a further embodiment of the method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans, the P-alpha-1,4-glucan-binding proteins dissolved according to process step b) i or the non-phosphorylated alpha-1,4-glucan-binding proteins dissolved according to process step b) ii are

separated from one another when implementing the method according to the invention.

The dissolved P-alpha-1,4-glucan-binding proteins or the dissolved non-phosphorylated alpha-1,4 glucan-binding proteins can be separated using methods known to the person skilled in the art such as, for example, gel filtration, chromatographic methods, electrophoresis etc. The P-alpha-1,4-glucan-binding dissolved proteins or the non-phosphorylated alpha-1,4 glucan-binding dissolved proteins are preferably separated from one another by means of SDS acrylamide gel electrophoresis, particularly preferably using the method described further below (see General Methods, Item 9).

A further object of the present invention is a method for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, wherein

a) protein extracts are incubated with phosphorylated alpha-1,4-glucans,

b) proteins specifically bound to the phosphorylated alpha-1,4-glucans from step a) are dissolved,

c) proteins obtained according to step b) are respectively incubated with

i) ATP and phosphorylated alpha-1,4-glucans and

ii) ATP and non-phosphorylated alpha-1,4-glucans

in preparations separated from one another,

d) the respective alpha-1,4-glucan obtained after incubation in step c) i or step c) ii is examined for introduction of further phosphate groups and

e) proteins are identified which in the incubation preparation according to c) i have introduced significant quantities of phosphate groups into alpha-1,4-glucans and in the incubation preparation according to c) ii have introduced no significant quantities of phosphate groups into alpha-1,4-glucans.

The term "elevated binding activity" should be understood in conjunction with the present invention as an increased affiinity of a protein to a first substrate compared to a second substrate; i.e. that the quantity of protein which under the same incubation conditions binds increasedly to a first substrate compared to a second substrate, exhibits an elevated binding activity to the first substrate.

The term "alpha-1,4-glucan" should be understood in conjunction with the present invention as a glucan which mainly consists of alpha-1,4-linked glucose building blocks but can also contain alpha-1,6-links as branches. An alpha-1,4-glucan preferably contains up to 15%, particularly preferably up to 10% and especially preferably up to 5% of alpha-1,6-links.

The term "starch phosphate" should be understood in conjunction with the present invention as phosphate groups covalently bound to the glucose molecules of an alpha-1,4-glucan.

The term "non-phosphorylated alpha-1,4-glucan" should be understood in conjunction with the present invention as an alpha-1,4- glucan which contains no detectable quantities of starch phosphate.

The term "phosphorylated alpha-1,4-glucan" or "P-alpha-1,4-glucan" should be understood in conjunction with the present invention as an alpha-1,4-glucan which contains starch phosphate.

Basically, a protein identifiable using a method according to the invention can come from any organism. The protein preferably comes from plant organisms, preferably from starch-storing plants (maize, rice, wheat, rye, oats, barley, cassava, potato, sweet potato, sago, mung bean, banana, pea, *Arabidopsis*, *Curcuma* or sorghum),

particularly preferably from potato, barley, sugar beet, *Arabidopsis* or rice plants and especially preferably *Arabidopsis* or rice plants.

In a further embodiment of the method according to the invention, the protein extracts come from eukaryotic cells, preferably from plant cells, particularly preferably from cells of starch-storing (maize, rice, wheat, rye, oats, barley, cassava, potato, sweet potato, sago, mung bean, banana, pea, arabidopsis, curcuma or sorghum) plants.

Basically all non-phosphorylated alpha-1,4-glucans are suitable for incubating protein extracts with non-phosphorylated alpha-1,4-glucans for implementing the method according to the invention. Preferably used is a non-phosphorylated plant starch, particularly preferably wheat starch and especially preferably granular leaf starch of the *Arabidopsis thaliana* mutant *sex1-3* (Tien-Shin Yu et al., 2001, Plant Cell 13, 1907-1918).

Methods for isolating starch from plants, for example, are known to the person skilled in the art. All methods known to the person skilled in the art are basically suitable for isolating non-phosphorylated starch from appropriate plant species. Preferably, the method for isolating non-phosphorylated alpha-1,4-glucans described below is used (see General Methods Item 2)

Basically all alpha-1,4-glucans containing starch phosphate are suitable for incubating protein extracts with P-alpha-1,4-glucans for implementing the method according to the invention. Chemically phosphorylated starches can also be used in this case. Preferably used for incubation with protein extracts are plant P-alpha-1,4-glucans, particularly preferably a subsequently enzymatically phosphorylated plant starch, especially preferably a subsequently enzymatically phosphorylated plant granular starch which was isolated from a *sex1-3* mutant of *Arabidopsis thaliana*.

A subsequent enzymatic phosphorylation of non-phosphorylated alpha-1,4-glucans can be carried out with any enzyme which transfers phosphate residues to non-phosphorylated alpha-1,4-glucans by introduction of covalent bonds. Preferably used for this purpose is an enzyme having the activity of a water glucan dikinase (R1 Protein, E.C.: 02.07.09.4) (Ritte et al., 2002, PNAS 99, 7166-7171; Mikkelsen et al., 2004, Biochemical Journal 377, 525-532). Preferably used for the subsequent enzymatic phosphorylation of non-phosphorylated alpha-1,4-glucans is a purified R1 protein, especially an R1 protein from potato produced by heterologous expression in *E. coli*.

Methods for purifying an R1 protein produced recombinantly by expression in *E. coli* are described in Ritte et al. (2002, PNAS 99, 7166-7171) and Mikkelsen et al. (2003, Biochemical Journal 377, 525-532).

When implementing the method according to the invention, P-alpha-1,4-glucan-protein complexes can be formed by incubation of protein extracts with P-alpha-1,4-glucans and/or non-phosphorylated alpha-1,4-glucans as a result of the binding of proteins to P-alpha-1,4-glucans and non-phosphorylated alpha-1,4-glucan-protein complexes can be formed as a result of the binding of proteins to non-phosphorylated alpha-1,4-glucans.

The proteins present in P-alpha-1,4-glucan-protein complexes or non-phosphorylated alpha-1,4-glucan-protein complexes when implementing the method according to the invention are dissolved, i.e., the binding of the proteins concerned to the respective alpha-1,4-glucans is broken. Dissolved P-alpha-1,4-glucan-binding proteins and/or dissolved non-phosphorylated alpha-1,4-glucan-binding proteins are thus obtained. Basically, all substances which prevent the existing protein-alpha-1,4-glucan interaction can be used to break the binding between the alpha-1,4-glucans concerned and the proteins bound to them. Preferred for this purpose are buffer solutions containing detergents, particularly preferably buffer solutions containing

sodium lauryl sulphate (SDS), especially preferably the buffer solution described further below (see General Methods Item 8).

Any method which allows alpha-1,4-glucans to be separated from the dissolved substances, such as proteins and, for example, ATP of the incubation preparation, can be used to separate alpha-1,4-glucans from ATP and/or proteins. If soluble alpha-1,4-glucans are used for the incubation of protein extracts with alpha-1,4-glucans when implementing the method according to the invention, the separation can, for example, involve a precipitation of the alpha-1,4-glucans, preferably a precipitation with suitable solvents, particularly preferably a precipitation with alcohols. The separation of alpha-1,4-glucans by binding to substances which selectively bind alpha-1,4-glucans (e.g. Concavalin A) is also suitable for separating alpha-1,4-glucans from substances in solution.

Preferably used here for the separation of alpha-1,4-glucans is filtration, particularly preferably centrifugation, especially preferably the method described further below (see General Methods Item 8).

When implementing the method according to the invention, all methods known to the person skilled in the art, such as chromatographic methods, for example, precipitation and subsequent centrifugation of the alpha-1,4-glucan, enzymatic digestion of the alpha-1,4-glucans, gel filtration etc. which lead to separation of soluble proteins from alpha-1,4-glucans, can basically be used to separate soluble proteins from the alpha-1,4-glucans. The dissolved P-alpha-1,4-glucan-binding proteins and/or dissolved non-phosphorylated alpha-1,4-glucan-binding proteins are preferably separated from the alpha-1,4-glucans used in the method according to the invention with the aid of centrifugation.

In a further embodiment of the present invention when implementing the method according to the invention, centrifugation using a Percoll pad is used to separate P-

alpha-1,4-glucan-protein complexes from proteins not contained in the complexes concerned.

The method described further below (see General Methods Item 8) is preferably used here to separate the proteins not bound to the alpha-1,4-glucans. After centrifugation has been carried out using a Percoll pad, the proteins not bound to P-alpha-1,4-glucans or not bound to non-phosphorylated alpha-1,4-glucans are located in the supernatant of the centrifugation medium whereas the P-alpha-1,4-glucan-protein complexes or non-phosphorylated alpha-1,4-glucan-protein complexes are present in the sedimented pellet. The supernatant of the centrifugation medium is discarded and the pellet is preferably washed with the buffer used for the incubation for further purification of the P-alpha-1,4-glucan-protein complexes or non-phosphorylated alpha-1,4-glucan-protein complexes. The pellet is preferably washed once, particularly preferably twice.

Basically any type of protein extract can be used to carry out the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate. Both so-called protein raw extracts and partly or completely purified protein extracts can be involved here. Thus, for example, it is advantageous to use proteins which were identified using a method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans. Proteins which were identified using a method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans, can, for example, be used omitting process steps a) and b) directly in step c) of the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate.

Basically, all general methods known to the person skilled in the art, such as described, for example, in Scopes (1993, Protein Purification: Principles & Practice, ISSN: 038794072) are suitable for producing protein extracts from prokaryotic or eukaryotic cells for implementing the method according to the invention. Preferably used for implementing the method, however, are methods for the isolation of plant proteins (e.g. described in Bollag et al, 1996, in: "Protein Methods", 2nd Edition, Wiley, ISBN: 0-471-11837-0; Dennison, 2003, in: "A Guide to Protein Isolation" 2nd Edition, Kluwer Academic Publishers, ISBN 1-4020-1224-1), particularly preferably the method described further below (see General Methods Item 1).

The incubation of protein extracts for implementing the method according to the invention with P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans takes place in separate preparations. The relevant preparations for P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans are treated separately from one another during the implementation of the entire method. In this case, respectively the same quantities of protein extract are to be incubated with respectively the same quantities of P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans. Preferably, respectively 1 to 10 mg, particularly preferably 3 to 7 mg and especially preferably 4 to 6 mg of protein extract are incubated with P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans. The quantity of P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans used is preferably respectively 10 to 100 mg, particularly preferably 30 to 70 mg and especially preferably 45 to 55 mg.

Various buffers can be used for the incubation of protein extracts with P-alpha-1,4-glucans for implementing the method according to the invention. Basically all buffers which allow binding of the proteins to be identified to the substrate concerned are suitable. The buffer described further below (see General Methods Item 1) is preferably used.

The term "protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate" should be

understood in conjunction with the present invention as a protein which introduces phosphate residues covalently into P-alpha-1,4-glucans, that is uses P-alpha-1,4-glucans as a substrate for the transfer of phosphate residues whereas non-phosphorylated P-alpha-1,4-glucans are not phosphorylated by a protein concerned, i.e., non-phosphorylated P-alpha-1,4-glucans do not serve as a substrate for a phosphorylation reaction.

In a further embodiment the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate relates to a method for identifying a protein which uses ATP as a further substrate.

In this embodiment of the present invention, ATP is used as a further substrate (co-substrate) by the protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, i.e. the protein concerned transfers a phosphate residue from ATP to an already phosphorylated P-alpha-1,4-glucan.

The activity of a protein which uses ATP as co-substrate for the transfer of phosphate residues to P-alpha-1,4-glucans can be demonstrated, i.e. by using ATP which contains a labeled phosphate residue (labeled ATP). To be preferred is ATP in which the phosphate residue is specifically labeled in the beta-position, i.e., in which only the phosphate residue in the beta-position has a marking. Preferably radioactively labeled ATP, particularly preferably ATP, in which the phosphate residue is specifically radioactively labeled in the beta position, and especially preferably ATP, in which the phosphate residue is specifically labeled with ^{33}P in the beta position, is used. If a P-alpha-glucan phosphorylating protein is incubated with P-alpha-1,4-glucans in the presence of labeled ATP, labeled phosphate covalently bound to the P-alpha-1,4-glucan can then be detected. In this case, the P-alpha glucans used for the phosphorylation reaction can be present both in the form of starch-phosphate-

containing plant starch (potato starch, starch from *Curcuma armada, C. zedoaria, C. longa*, rice, mung beans, tapioca etc) and also in the form of enzymatically phosphorylated P-alpha-1,4-glucans or chemically phosphorylated P-alpha-1,4-glucans. Preferably starch from leaves of *Arabidopsis thaliana*, particularly preferably starch from *Arabidopsis thaliana sex1-3* mutants enzymatically phosphorylated by means of an R1 protein is used.

Labeled phosphate residues which can be incorporated into a P-alpha-1,4-glucan by a protein, e.g., after separation of the labeled P-alpha-1,4-glucan (e.g., by precipitation of the alpha-1,4-glucans by means of ethanol, filtration, chromatographic methods, centrifugation etc.) from the remainder of the reaction mixture and subsequent detection of the labeled phosphate residues in the relevant P-alpha-1,4-glucan fraction, can be demonstrated. At the same time, the labeled phosphate residues bound in the P-alpha-1,4-glucan fraction can be demonstrated, for example, by determining the amount of radioactivity present in the P-alpha-1,4-glucan fraction (e.g. by means of scintllation counters).

In a further embodiment, the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate relates to a method wherein the protein having alpha-1,4-glucan phosphorylating enzymatic activity uses P-starch as substrate. Starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana*, which was subsequently enzymatically phosphorylated is particularly preferred. For implementing this preferred embodiment of the method according to the invention, a phosphorylated starch is accordingly used in the process steps c) i and a non-phosphorylated starch is used in process step c) ii.

It is thereby possible to identify proteins which phosphorylate P-starch. Such proteins are especially suitable for modifying starch in plant organisms by means of genetic manipulation of appropriate plants.

In a further embodiment the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate relates to a method for identifying a protein wherein the protein occurs as a phosphorylated intermediate product during the transfer of a phosphate residue to a P-alpha-1,4-glucan. Said intermediate product is preferably formed by autophosphorylation of the protein concerned.

A phosphorylated protein which occurs as an intermediate product as a result of protein-mediated phosphorylation of P-alpha-1,4-glucans can be demonstrated as described in Ritte et al. (2002, PNAS 99, 7166-7171) for an R1 protein.

In order to detect the presence of an autophosphorylated intermediate product, a protein is first incubated in the absence of glucans with labeled ATP, preferably with ATP labeled specifically in the beta phosphate position, particularly preferably with ATP labeled specifically with ^{33}P in the beta phosphate position for 15 to 45 minutes, particularly preferably for 20 to 40 minutes and especially preferably for 25 to 30 minutes in a reaction preparation 1. Parallel to this, a reaction preparation 2 which contains corresponding quantities of non-labeled ATP instead of labeled ATP, is incubated under otherwise the same conditions. Non-labeled ATP is then added to reaction mixture 1 in excess and a mixture of non-labeled ATP and labeled ATP (the same quantity of labeled ATP as used previously in reaction mixture 1 and the same quantity of non-labeled ATP as added in excess to reaction mixture 1) is added to reaction mixture 2 and incubated for a further 1 minute to 5 minutes, preferably for 2 to 5 minutes and especially preferably for 3 minutes before P-alpha-1,4-glucans are added to a Part A of reaction mixture 1 (Part 1A) or to a Part A of reaction mixture 2 (Part 2A). The reaction in the remaining Part 1B and Part 2B of the reaction mixture is stopped by denaturing the protein. Part B of the reaction mixture can be stopped by the methods known to the person skilled in the art, which lead to the denaturing of proteins, preferably by adding sodium lauryl sulphate (SDS). Part 1A and Part 2A of

the reaction mixtures are incubated for at least a further 10 minutes before these reactions are also stopped. The alpha-1,4-glucans present in Part A or Part B of the respective reaction mixtures are separated from the respective remainder of the reaction mixtures. If the respective alpha-1,4-glucans are separated by centrifugation, for example, then, on completion of centrifugation, the alpha-1,4-glucans of the respective Part A or Part B of the reaction mixture are to be found in the sedimented pellet, and the proteins in the respective reaction mixtures are to be found in the supernatant of the respective centrifugation. The supernatant of Part 1A or 2A and of Part 1B or 2B of the reaction mixture can then be analysed, for example, respectively in a denaturing acrylamide gel electrophoresis, followed by autoradiography of the acrylamide gel obtained. To quantify the amount of radioactively labeled proteins, which have been separated by means of acrylamide gel electrophoresis, the so-called "phospho-imaging" method, for example, known to the person skilled in the art, can be used. If the autoradiography or the analysis by means of the "phospho-imager" of proteins in the centrifugation supernatant of Part B of reaction mixture 1 shows a significantly increased signal compared with the centrifugation supernatant of Part A of reaction mixture 1, then this shows that a protein mediating a phosphorylation of alpha-glucans occurs as an autophosphorylated intermediate product. Parts A and B of reaction mixture 2 serve as a control and should therefore not exhibit a significantly increased signal in the centrifugation supernatant in the autoradiography or in the analysis by means of the "phospho-imager".

In addition, the alpha-1,4-glucans of the respective Part A of reaction mixtures 1 and 2 remaining in the respective sedimented pellet can be investigated, if necessary after subsequent washing of the respective alpha-1,4-glucans, for the presence of starch phosphate, which has a mark corresponding to the labeled ATP used. If the alpha-1,4-glucans of Part A of reaction mixture 1 contain labeled phosphate residues, and if the autoradiography of the centrifugation supernatant of Part B of reaction mixture 1 shows a significantly increased signal in the autoradiography compared with the centrifugation supernatant of Part A of reaction mixture 1, then this shows that a protein mediating a phosphorylation of alpha-glucans is present as an

autophosphorylated intermediate product. Parts A and B of reaction mixture 2 serve as a control and should therefore not exhibit a significantly increased signal for alpha-1,4-glucans labeled with ^{33}P in the sedimented pellet containing alpha-1,4-glucans.

In a further embodiment the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate relates to a method for identifying a protein which preferably introduces phosphate monoester bonds in the C-2 position or in the C-3 position, particularly preferably in the C-3 position of a glucose molecule of a P-alpha-1,4-glucan.

Which positions of the carbon atoms (C-2, C-3 or C-6) of the glucose monomers in the P-alpha-1,4-glucan are preferably phosphorylated by a protein or protein extract can be determined, for example, by analysing the P-alpha-1,4-glucans phosphorylated by a protein or protein extract, as described in Ritte et al. (2002, PNAS 99, 7166-7171). For this purpose P-alpha-1,4-glucans additionally phosphorylated by a protein or protein extract are hydrolysed using acid and then analysed by means of anion exchange chromatography.

The P-alpha-1,4-glucans phosphorylated by a protein are preferably analysed by means of NMR in order to determine which positions of the carbon atoms (C-2, C-3 or C-6) of the glucose monomers are phosphorylated in P-alpha-1,4-glucan.

Proteins of the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, which were obtained according to process step b) are incubated in step c) of the method according to the invention in separate preparations containing ATP and P-alpha-1,4-glucan or ATP and non-phosphorylated alpha-1,4-glucan. For implementing the method according to the invention it is preferable to use ATP which contains a labeled phosphate residue,

particularly preferably a phosphate residue specifically labeled in the beta position, especially a phosphate residue specifically radioactively labeled in the beta position.

The incubation of dissolved proteins according to the invention with ATP and P-alpha-1,4-glucans according to process step c) i or non-phosphorylated alpha-1,4-glucans according to step c) ii of the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, preferably takes place at a temperature of 20°C to 30°C, particularly preferably 23°C to 27°C and especially preferably 24°C to 26°C and is carried out for a duration of at least 15 minutes, preferably for at least 20 minutes, particularly preferably for at least 30 minutes. The quantity of ATPs used in this case is preferably at least 0.05 µM, particularly preferably at least 3 µM and especially preferably at least 5 µM. The concentration of the P-alpha-1,4-glucan used or the non-phosphorylated alpha-1,4-glucan used is in this case preferably at least 1 mg/ml, particularly preferably at least 10 mg/ml and especially preferably at least 25 mg/ml. After incubation has been completed, the reactions of protein extracts with P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans can be stopped. The respective reaction mixture can be stopped by methods known to the person skilled in the art which lead to denaturing of proteins, preferably by adding sodium lauryl sulphate and heating for 5 minutes at 95°C. When implementing step c) i or c ii) of the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, respectively the same incubation conditions for the respective incubation preparations should be carried out during the incubation of proteins with P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans.

The P-alpha-1,4-glucan obtained according to process step c) i or the non-phosphorylated alpha-1,4-glucan obtained according to process step c) ii after implementing the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires

phosphorylated alpha-1,4-glucans as substrate, is investigated for the introduction of additional phosphate residues. In order to determine whether phosphate residues were additionally introduced into the alpha-1,4-glucans concerned by process steps c) i and/or c) ii, any method which is possible for the specific detection of the marking used for the labeled ATPs used in process steps c) i and c) ii can be used. If, for example, radioactively labeled ATP is used in process steps c) i or c) ii,, this can be carried out using methods known to the person skilled in the art for the detection of radioactive elements, such as, for example, autoradiography, measurement of the radioactivity by means of suitable equipment (e.g. scintillation counters, "phospho-imagers" etc.).

Proteins used in process step b) of the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, which have introduced significant quantities of phosphate residues into P-alpha-1,4-glucans in step c) i but in comparison thereto, have introduced no significant quantities of phosphate residues into non-phosphorylated alpha-1,4-glucans in step c) ii, can be identified by methods known to the person skilled in the art.

The term "significant quantities" should be understood in conjunction with the present invention as a quantity which is at least twice, preferably at least four times, particularly preferably at least six times and especially preferably at least eight times higher than the quantity determined in corresponding control experiments.

In this case, incubation preparations which contain completely inactivated protein extracts or no protein extracts instead of native protein extracts can be used as control experiments. Protein extracts in which no more alpha-1,4-glucan phosphorylating enzymatic activity can be detected are to be understood as "completely inactivated".

Identifying proteins when implementing the method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans can be made using methods known to the person skilled in the art such as, for example, determining the amino acid sequence of the proteins concerned using methods comprising Edmann degradation, mass analysis using MALDI-TOF-MS (Matrix Assisted Laser Desorption/Ionization-Time Of Flight-Mass Spectroscopy), followed by comparisons with data bases containing mass profiles of proteins, amino acid sequencing by means of Q-TOF analysis or TOF/TOF analysis etc. The proteins concerned are preferably identified by means of Q-TOF-MS-MS analysis, especially preferably the proteins are identified using the method described further below (see General Methods Item 10).

If proteins are determined by means of MALDI-TOF-MS, followed by comparisons with databases containing mass profiles of proteins, the proteins concerned are first enzymatically digested beforehand before the individual masses of the protein fragments (peptides) obtained from the digestion are analysed by means of MALDI-TOF-MS. A mass profile of the protein concerned is obtained. These mass profiles are very specific for a protein since sequence-specific proteases are used for the digestion of proteins which only cleave a peptide bond when it is contained in a specific amino acid sequence succession. If the special amino acid sequence which serves as a recognition sequence for a certain protease is known, a theoretical mass profile can be created from any arbitrary amino acid sequence by calculating the mass of the peptides which would be produced after digestion of the amino acid sequence with a specific protease. By comparing mass profiles of unknown proteins actually obtained using MALDI-TOF-MS with the theoretically determined mass profiles in corresponding databases, amino acid sequences can thus also be determined.

In a further embodiment the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and

requires phosphorylated alpha-1,4-glucans as substrate, the P-alpha-1,4-glucan-protein complexes obtained by incubating protein extracts with P-alpha-1,4-glucans according to step a) are separated from the proteins not bound to the alpha-1,4-glucans concerned.

In a further embodiment of the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, the proteins dissolved according to step b) of the method according to the invention are separated from the P-alpha-1,4-glucans used in step a).

In a further embodiment the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, the dissolved P-alpha-1,4-glucan-binding proteins, obtained when implementing the method according to the invention according to process step b), are separated from one another.

In a further embodiment of the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, the glucans obtained by incubation of protein extracts with P-alpha-1,4-glucans according to step c) i or with non-phosphorylated alpha-1,4-glucans according to step c) ii are separated from the proteins present in the reaction mixture and/or the labeled ATP present in the reaction mixture.

Preferably used here for the separation of alpha-1,4-glucans is filtration, particularly preferably centrifugation, especially preferably the method described further below (see General Methods Item 8). After centrifugation has been carried out using a Percoll pad, soluble substances of the reaction mixtures are located in the

Thus, proteins obtainable by methods according to the invention for identifying a protein are also the object of the present invention.

A method for identifying a nucleic acid coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity is further an object of the present invention, wherein

a) a protein is identified using a method according to the invention for identifying a protein,

b) amino acid sequences coding for the protein identified according to step a) are determined and

c) nucleic acids are identified using the amino acids determined according to step b), which code for a protein identified according to step a).

The amino acid sequence of the proteins identified using a method according to the invention can be determined using methods known to the person skilled in the art, as already stated above.

On the basis of the amino acid sequences determined according to step b) of the method according to the invention for identifying a nucleic acid, coding for a protein which exhibits alpha-1,4-glucan-phosphorylating enzymatic activity, nucleic acids coding for a protein exhibiting alpha-1,4-glucan-phosphorylating enzymatic activity can be identified.

Nucleic acids coding for a protein exhibiting alpha-1,4-glucan-phosphorylating enzymatic activity can be identified, for example, by scrutinising databases such as those made available, for example by EMBL (http://www.ebi.ac.uk/Tools/index.htm) or NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). In this case one or a plurality of amino acid sequences determined when implementing the method according to the invention, is pre-defined

as a so-called query. This query sequence is then compared by means of statistical computer programs with sequences, which are contained in the selected databases. Such database queries (e.g. blast or fasta searches) are known to the person skilled in the art and can be carried out by various providers.

If such a database query is carried out, e.g. at the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/), then the standard settings, which are specified for the particular comparison inquiry, should be used. For protein sequence comparisons (blastp), these are the following settings: Limit entrez = not activated; Filter = low complexity activated; Expect value = 10; word size = 3; Matrix = BLOSUM62; Gap costs: Existence = 11, Extension = 1.

During such a database search, for example, the amino acid sequences determined in the present invention when implementing the method according to the invention can be used as a query sequence in order to identify nucleic acid molecules coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity.

Using the method described, it is also possible to identify nucleic acid molecules and/or amino acid sequences which have a high degree of identity to nucleic acid molecules and/or proteins obtainable using the method according to the invention and coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity.

Methods are known to the person skilled in the art with which, starting from amino acid sequences, he can identify nucleic acids coding for these (see, for example, Sambrok et al., Molecular Cloning, A Laboratory Manual, 3rd edition (2001) Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. ISBN: 0879695773, Ausubel et al., Short Protocols in Molecular Biology, John Wiley & Sons; 5th edition (2002), ISBN: 0471250929). From amino acid sequences coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity, nucleic acids coding for the amino acid sequences concerned can be derived in accordance with the genetic

code. It is known to the person skilled in the art that the degenerated oligonucleotides obtained from the genetic code can basically also be used to identify nucleic acids. Oligonucleotides which constitute sequences derived from the amino acid sequences obtained when implementing the method according to the invention can then be synthesised. These synthetic oligonucleotides can be used to identify nucleic acids coding for the proteins from whose amino acid sequence the corresponding oligonucleotide sequences were derived. This can be achieved, for example, by searching gene libraries, said synthetic oligonucleotides being used as labeled probes in the form of hybridisation probes. A further possibility for identifying nucleic acids coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity involves using the synthetic oligonucleotides derived from amino acid sequences obtained when implementing the method according to the invention, by searching gene libraries using PCR based methods, wherein said synthetic oligonucleotides are used as so-called "primers". Gene libraries can be present, for example, in the form of cosmids, phagmids, plasmids, YACs or BACs. The DNA libraries can contain both genomic and also cDNA. For PCR-based searching methods when using the so-called RT (Reverse Transcription) PCR, it is also possible to use mRNA The nucleic acids for the implementation of the method according to the invention for identifying a nucleic acid in gene libraries or present as mRNA can in this case come from any organism, preferably they come from eukaryotic, particularly preferably from plants, especially preferably from cereals.

For the implementation of the method according to the invention for identifying a nucleic acid coding for a protein which exhibits alpha-1,4-glucan-phosphorylating enzymatic activity, it is not necessary that the entire amino acid sequence coding for the protein concerned is determined in step b) of the method according to the invention but it can be sufficient if only parts of the amino acid sequences concerned, coding for a protein concerned, are determined.

A further embodiment of the present invention relates to a method for identifying a nucleic acid coding for a protein which exhibits alpha-1,4-glucan-phosphorylating enzymatic activity, wherein

a) a protein is identified using a method according to the invention for identifying a protein,

b) amino acid sequences coding for the protein identified according to step a) are determined

c) oligonucleotides are synthesised starting from the amino acid sequences determined in step b) and

d) nucleic acids coding for a protein identified according to step a) are identified with the aid of the oligonucleotides synthesised according to step c)

A further object of the present invention relates to a method for identifying a nucleic acid coding for a protein which exhibits alpha-1,4-glucan-phosphorylating enzymatic activity, wherein

a) a protein is identified using a method according to the invention for identifying a protein,

b) antibodies which react specifically with the protein identified according to step a) are produced and

c) nucleic acids are identified using the antibodies determined according to step b).

Methods for manufacturing antibodies, which react specifically with a certain protein, i.e. which bind specifically to said protein, are known to the person skilled in the art (see, for example, Lottspeich and Zorbas (Eds.), 1998, Bioanalytik, Spektrum akad, Verlag, Heidelberg, Berlin, ISBN 3-8274-0041-4). The manufacture of such antibodies is offered by some companies (e.g. Eurogentec, Belgium) as a contract service.

Methods for identifying nucleic acids using antibodies, frequently designated as "immunoscreening" in the specialist literature (see, for example Lottspeich und Zorbas (Eds.), 1998, Bioanalytik, Spektrum akad. Verlag., Heidelberg, Berlin, ISBN 3-8274-0041-4) are likewise known to the person skilled in the art and described in detail in the literature. So-called expression gene libraries, for example, can be used to implement such methods, in which the clones obtained are seaached for the expression of a certain protein with the aid of a specific antibody directed against this protein. Materials for manufacturing such expression gene libraries, also containing instructions relating the method for the manufacture and also methods for searching such expression gene banks can be purchased (e.g. Stratagene).

Using methods according to the invention, it is possible to identify nucleic acids coding for proteins which exhibit elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans and/or which exhibit alpha-1,4-glucan phosphorylating enzymatic activity and require phosphorylated alpha-1,4-glucans as substrate.

Thus, nucleic acids obtainable by methods according to the invention for identifying a nucleic acid, are also the object of the present invention.

A plasmid (A.t.-OK1-pGEM) containing a cDNA which codes for a protein according to the invention (A.t.-OK1) from *Arabidopsis thaliana* was deposited on 08.03.2004 under the number DSM16264 and a plasmid (pM150) containing a cDNA which codes for further protein according to the invention (O.s.-OK1) from *Oryza sativa* was deposited on 24.03.2004 under the number DSM16302 under the Budapest Treaty at the German Collection of Microorganisms and Cell Cultures GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany.

It was surprisingly found that genetically modified plant cells or plants which exhibit an elevated activity of a protein according to the invention, synthesise a modified

starch which is modified in its physical-chemical properties, especially the content of starch phosphate or the phosphate distribution compared to starch synthesised in wild type plant cells or wild type plants so that this is better suited for special applications.

Thus, a further object of the present invention relates to genetically modified plant cells or genetically modified plants characterised in that they exhibit an elevated enzymatic activity of a protein according to the invention compared to corresponding non-genetically modified wild type plant cells or wild type plants .

In this case, the genetic modification can be any genetic modification, which leads to an increase in the activity of at least one protein according to the invention in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

In conjunction with the present invention, the term "wild type plant cell" means that the plant cells concerned were used as starting material for the manufacture of the plant cells according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant cell according to the invention.

In conjunction with the present invention, the term "wild type plant " means that the plants concerned were used as starting material for the manufacture of the plants according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant according to the invention.

In conjunction with the present invention, the term "corresponding" means that, in the comparison of several objects, the objects concerned that are compared with one another have been kept under the same conditions. In conjunction with the present

invention, the term "corresponding" in conjunction with wild type plant cell or wild type plant means that the plant cells or plants, which are compared with one another, have been raised under the same cultivation conditions and that they have the same (cultivation) age.

The term "elevated activity" in the framework of the present invention means in this case an increase in the expression of endogenous genes coding for proteins according to the invention and/or an increase in the quantity of proteins according to the invention in the cells and/or an increase in the enzymatic activity of proteins according to the invention in the cells.

The increase in the expression can, for example, be determined by measuring the quantity of transcripts coding for proteins according to the invention, e.g. using Northern blot analysis or RT-PCR. Nucleic acid molecules which were identified using methods according to the invention for identifying a nucleic acid are preferably used in this case to determine an elevated expression of proteins according to the invention. Here, an increase preferably means an increase in the amount of transcripts in comparison with corresponding cells that have not been genetically modified by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 100%. An increase in the quantity of transcripts coding for a protein according to the invention also means that plants which have no detectable transcripts coding for a protein according to the invention, after genetic modification according to the invention, have a detectable quantity of transcripts coding for a protein according to the invention.

The increase in the amount of protein of a protein according to the invention, which results in an increased activity of this protein in the plant cells concerned, can, for example, be determined by immunological methods such as Western Blot analysis, ELISA (Enzyme Linked Immuno Sorbent Assay) or RIA (Radio ImmunoAssay). The manufacture of an antibody which can be used to measure the increase in the amount of protein using immunological methods is described further below as an

example (see Example 11). Here, an increase preferably means an increase in the amount of a protein according to the invention in comparison with corresponding cells that have not been genetically modified by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 100%. An increase in the amount of a protein according to the invention also means that plants which have no detectable amount of a protein according to the invention, after genetic modification according to the invention, have a detectable amount of protein according to the invention.

It was surprisingly also found that genetically modified plant cells or plants which exhibit a reduced activity of a protein according to the invention, synthesise a modified starch which is modified in its physical-chemical properties, especially relating to the phosphate distribution compared to starch synthesised in wild type plant cells or wild type plants so that this is better suited for special applications.

Thus, a further object of the present invention relates to genetically modified plant cells or genetically modified plants, characterised in that they exhibit a reduced enzymatic activity of a protein according to the invention compared to corresponding wild type plant cells or wild type plants which have not been genetically modified .

Plants which exhibit a reduced activity of a protein according to the invention, exhibit a high starch (starch excess) phenotype. Furthermore, plants which exhibit a reduced activity of a protein according to the invention, exhibit normal growth compared to wild type plants, i.e., the plants are not hindered in their growth by the reduced activity of a protein according to the invention.Therefore, plants which exhibit a reduced activity of a protein according to the invention, are suitable for cultivation in agriculture since they contain more starch and therefore more carbohydrate and at the same time show no reduction in growth rate.

The present invention therefore also relates to plant cells and plants according to the invention which exhibit a starch excess phenotype. Plant cells according to the invention and plants according to the invention have at least twice, preferably at least four times, particularly preferably at least six times and especially preferably at least eight times more starch in their leaves at the end of the dark phase than corresponding wild type plant cells or wild type plants.

Plant cells according to the invention and plants according to the invention have at least 1.2 times, preferably at least 1.5 times, particularly preferably at least 1.8 times and especially preferably at least twice more starch in their leaves at the end of the light phase than corresponding wild type plant cells or wild type plants.

The plant cells according to the invention and plants according to the invention which exhibit a reduced activity of a protein according to the invention, can be manufactured by various methods known to the person skilled in the art. These include, for example, the expression of a corresponding antisense RNA, or a double-stranded RNA construct, the preparation of molecules or vectors which impart a co-suppression effect, the expression of a correspondingly constructed ribozyme which specifically cleaves transcripts which code for a protein according to the invention or the so-called "*in vivo* mutagenesis". Moreover, the reduction of the activity of a protein according to the invention in plant cells and plants can also be brought about by the simultaneous expression of sense and antisense RNA molecules of the respective target gene to be repressed, preferably the OK1 gene.

It is additionally known that the *in planta* formation of double-stranded RNA molecules of promoter sequences *in trans* can lead to a methylation and a transcriptional inactivation of homologous copies of this promoter (Mette et al., EMBO J. 19, (2000), 5194-5201).

Another possible method for reducing the enzymatic activity of proteins in plant cells or plants is the so-called immunomodulation method. It is known that an *in planta* expression of antibodies which specifically recognise a plant protein results in a reduction in the activity of the relevant protein in corresponding plant cells as a result of the formation of a protein antibody complex (Conrad and Manteufel, Trends in Plant Science 6, (2001), 399-402; De Jaeger et al., Plant Molecular Biology 43, (2000), 419-428; Jobling et al., Nature Biotechnology 21, (2003), 77-80).

All these methods are based on the introduction of a foreign or a plurality of foreign nucleic acid molecules into the genome of plant cells or plants and are therefore fundamentally suitable for manufacturing plant cells according to the invention and plants according to the invention.

In a further embodiment of the present invention, the plant cells according to the invention or plants according to the invention comprise plant cells of starch-storing plants or starch-storing plants. Starch-storing plants are, for example, maize, rice, wheat, rye, oats, barley, cassava, potato, sweet potato, sago, mung bean, banana, pea, *Arabidopsis*, curcuma or sorghum plants. Particularly preferred are rice, especially preferred are wheat plants.

A further embodiment of the present invention relates to a genetically modified plant cell according to the invention or a genetically modified plant according to the invention, wherein the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant.

In this context, the term "genetic modification" means the introduction of homologous and/or heterologous foreign nucleic acid molecules into the genome of a plant cell or into the genome of a plant, wherein said introduction of these molecules leads to an increase or reduction in the activity of a protein according to the invention.

The plant cells according to the invention or plants according to the invention are modified with regard to their genetic information by the introduction of a foreign nucleic acid molecule. The presence or the expression of the foreign nucleic acid molecule leads to a phenotypic change. "Phenotypic" change preferably means in this case a measurable change in one or a plurality of functions of the cells. For example, the genetically modified plant cells according to the invention and the genetically modified plants according to the invention exhibit an increase or reduction in the activity of a protein according to the invention due to the presence or on the expression of the introduced nucleic acid molecule.

In conjunction with the present invention, the term "foreign nucleic acid molecule" is understood to mean such a molecule that either does not occur naturally in the corresponding wild type plant cells, or that does not occur naturally in the specific spatial arrangement in wild type plant cells, or that is localised at a place in the genome of the wild type plant cell at which it does not occur naturally. Preferably, the foreign nucleic acid molecule is a recombinant molecule, which consists of different elements, the combination or specific spatial arrangement of which does not occur naturally in plant cells.

In principle, the foreign nucleic acid molecule can be any nucleic acid molecule, which effects an increase in the activity of a protein according to the invention in the plant cell or plant.

In conjunction with the present invention, the term "genome" is to be understood to mean the totality of the genetic material present in a plant cell. It is known to the person skilled in the art that, as well as the cell nucleus, other compartments (e.g. plastids, mitochondria) also contain genetic material.

A preferred embodiment of the present invention relates to a genetically modified plant cell according to the invention or a genetically modified plant according to the

invention, wherein the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant and the foreign nucleic acid molecule codes for a protein according to the invention.

A further embodiment of the present invention relates to a genetically modified plant cell according to the invention or a genetically modified plant according to the invention, wherein the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant and wherein the foreign nucleic acid molecule comprises a nucleic acid molecule according to the invention, preferably a nucleic acid molecule according to the invention, isolated from *Arabidopsis thaliana*, particularly preferably isolated from rice.

A large number of techniques are available for the introduction of DNA into a plant host cell. These techniques include the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as the transformation medium, the fusion of protoplasts, injection, the electroporation of DNA, the introduction of DNA by means of the biolistic approach as well as other possibilities.

The use of agrobacteria-mediated transformation of plant cells has been intensively investigated and adequately described in EP 120516; Hoekema, in: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and by An et al. EMBO J. 4, (1985), 277-287. For the transformation of potato, see Rocha-Sosa et al., EMBO J. 8, (1989), 29-33, for example.

The transformation of monocotyledonous plants by means of vectors based on *Agrobacterium* transformation has also been described (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al, Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153

(1992), 550-555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). 153 (1992), 550-555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). An alternative system to the transformation of monocotyledonous plants is transformation by means of the biolistic approach (Wan and Lemaux, Plant Physiol. 104, (1994), 37-48; Vasil et al., Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, electroporation of partially permeabilised cells and the introduction of DNA by means of glass fibres. In particular, the transformation of maize has been described in the literature many times (cf. e.g. WO95/06128, EP0513849, EP0465875, EP0292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726).

The successful transformation of other types of cereal has also already been described, for example, for barley (Wan and Lemaux, see above; Ritala et al., see above; Krens et al., Nature 296, (1982), 72-74) and for wheat (Nehra et al., Plant J. 5, (1994), 285-297). All the above methods are suitable within the framework of the present invention.

Amongst other things, the plant cells according to the invention and the plants according to the invention can be differentiated from wild type plant cells and wild type plants respectively in that they contain a foreign nucleic acid molecule, which does not occur naturally in wild type plant cells or wild type plants, or in that such a molecule is present integrated at a place in the genome of the plant cell according to the invention or in the genome of the plant according to the invention at which it does not occur in wild type plant cells or wild type plants, i.e. in a different genomic environment. Furthermore, plant cells according to the invention and plants according to the invention of this type differ from wild type plant cells and wild type plants respectively in that they contain at least one copy of the foreign nucleic acid molecule stably integrated within their genome, possibly in addition to naturally occurring copies of such a molecule in the wild type plant cells or wild type plants. If the foreign

nucleic acid molecule(s) introduced into the plant cells according to the invention or into the plants according to the invention is (are) additional copies of molecules already occurring naturally in the wild type plant cells or wild type plants respectively, then the plant cells according to the invention and the plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively in particular in that this additional copy or these additional copies is (are) localised at places in the genome at which it does not occur (or they do not occur) in wild type plant cells or wild type plants. This can be verified, for example, by using a Southern blot analysis.

Furthermore, the plant cells according to the invention and plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively preferably by at least one of the following features: If the foreign nucleic acid molecule that has been introduced is heterologous with respect to the plant cell or plant, then the plant cells according to the invention or plants according to the invention have transcripts of the introduced nucleic acid molecules. These can be verified, for example, by Northern blot analysis or by RT-PCR (Reverse Transcription Polymerase Chain Reaction). Preferably, the plant cells according to the invention and the plants according to the invention which exhibit an elevated activity of a protein according to the invention, contain a protein, which is coded for by an introduced nucleic acid molecule. This can be demonstrated by immunological methods, for example, in particular by a Western blot analysis. Plant cells according to the invention and plants according to the invention which exhibit a reduced activity of a protein according to the invention, show a reduced quantity of the relevant protein compared to corresponding wild type plant cells or wild type plants which have not been genetically modified, when investigated using said immunological methods.

If the foreign nucleic acid molecule that has been introduced is homologous with respect to the plant cell or plant, the plant cells according to the invention or plants according to the invention can be differentiated from wild type plant cells or wild type

plants respectively due to the additional expression of the introduced foreign nucleic acid molecule, for example. The plant cells according to the invention and the plants according to the invention preferably contain transcripts of the foreign nucleic acid molecules. This can be demonstrated by Northern blot analysis, for example, or using so-called quantitative PCR.

In a special embodiment, the plant cells according to the invention and the plants according to the invention are transgenic plant cells or transgenic plants respectively

In a further embodiment of the present invention, plant cells according to the invention and plants according to the invention synthesise a modified starch compared to starch isolated from wild type plant cells or wild type plants which have not been genetically modified.

In conjunction with the present invention, the term "modified starch" means that the starch has changed physical-chemical characteristics compared with non-modified starch obtainable from corresponding wild type plant cells or wild type plants.

In a further embodiment, the plant cells according to the invention or the plants according to the invention synthesise a modified starch which has an elevated content of starch phosphate and/or a modified phosphate distribution compared with starch isolated from corresponding wild type plant cells or wild type plants.

In a further embodiment of the method according to the present invention, the plant cells according to the invention or the plants according to the invention synthesise a modified starch which has a modified C-3/C-6 ratio of the starch phosphate compared with corresponding wild type plants cells which have not been genetically modified or plants which have not been genetically modified. Especially preferred in this case are starches which exhibit an elevated fraction of starch phosphate bound

in the C-3 position compared with starch phosphate bound in the C-6 position, in comparison to corresponding starches isolated from wild type plant cells which have not been genetically modified or plants which have not been genetically modified.

In conjunction with the present invention, the term "phosphate distribution" should be understood as the fraction of the starch phosphate bound in the C-2 position, C-3 position or C-6 position of a glucose molecule relative to the total starch phosphate content of alpha-1,4-glucans.

In conjunction with the present invention, the term "C-2/C-3/C-6 ratio" should be understood as the fraction of the starch phosphate in which the starch phosphate of an alpha-1,4-glucan bound respectively in the C-2 position, C-3 position or C-6 position contributes to the total starch phosphate content of the alpha-1,4-glucan concerned (C-2 position + C-3 position + C-6 position).

In conjunction with the present invention, the term "C-3/C-6 ratio" should be understood as the fraction of the starch phosphate in which the starch phosphate of an alpha-1,4-glucan bound respectively in the C-3 position and in the C-6 position contributes to the sum of the starch phosphate bound in the C-3 position and in the C-6 position (C-3 position + C-6 position) of the alpha-1,4-glucan concerned.

A further object of the present invention is plant cells according to the invention or plants according to the invention which synthesise a modified starch, wherein the modified starch is characterised in that it has an elevated content of phosphate covalently bound to the starch in the C-3 position of the glucose molecule compared to starch from corresponding wild type plant cells or wild type plants.

A further object of the present invention is plants containing plant cells according to the invention.

Description of sequences

SEQ ID NO 1: Nucleic acid sequence containing the coding region of the A.t.-OK1 protein from *Arabidopsis thaliana*. This sequence is inserted in the vectors OK1-pGEM-T and OK1-pDEST™17.

SEQ ID NO 2: Amino acid sequence coding for the A.t.-OK1 protein from *Arabidopsis thaliana*. This sequence can be derived from the nucleic acid sequence shown under SEQ ID NO 1.

SEQ ID NO 3: Nucleic acid sequence containing the coding region of the O.s.-OK1 protein from *Oryza sativa*. This sequence is inserted in the vector MI50.

SEQ ID NO 4: Amino acid sequence coding for the O.s.-OK1 protein from *Oryza sativa*. This sequence can be derived from the nucleic acid sequence shown under SEQ ID NO 3.

SEQ ID NO 5: Peptide sequence coding for the phosphohistidine domain of the OK1 proteins from *Arabidopsis thaliana*, *Oryza sativa* and *Sorghum bicolor*.

SEQ ID NO 6: Peptide sequence contained in the amino acid sequence coding for an H.v.-OK1 protein from barley.

SEQ ID NO 7: Peptide sequence contained in the amino acid sequence coding for an H.v.-OK1 protein from barley.

SEQ ID NO 8: Peptide sequence contained in the amino acid sequence coding for an H.v.-OK1 protein from barley.

SEQ ID NO 9: Partial nucleic acid sequence coding for an H.v.-OK1 protein from barley. This nucleic acid sequence has been identified by means of the peptide sequences shown under SEQ ID NO 6, SEQ ID NO 7 and SEQ ID NO 8 using the "Blast search" facility in the TIGR database.

SEQ ID NO 10: Partial amino acid sequence coding for an H.v.-OK1 protein from barley. The amino acid sequence shown can be derived from the nucleic acid sequence shown under SEQ ID NO 9.

SEQ ID NO 11: Peptide sequence contained in the amino acid sequence coding for an S.t.-OK1 protein from potato.

SEQ ID NO 12: Peptide sequence contained in the amino acid sequence coding for an S.t.-OK1 protein from potato.

SEQ ID NO 13: Peptide sequence contained in the amino acid sequence coding for an S.t.-OK1 protein from potato.

SEQ ID NO 14: Peptide sequence contained in the amino acid sequence coding for an S.t.-OK1 protein from potato.

SEQ ID NO 15: Partial nucleic acid sequence coding an S.t.-OK1 protein from potato. This nucleic acid sequence has been identified by means of the peptide sequences shown under SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13 and SEQ ID NO 14 using the "Blast Search" facility in the TIGR database.

SEQ ID NO 16: Partial amino acid sequence coding for an S.t.-OK1 protein from potato. The amino acid sequence shown can be derived from the nucleic acid sequence shown under SEQ ID NO 15.

SEQ ID NO 17: Peptide sequence contained in the amino acid sequence coding for an S.b.-OK1 protein from millet.

SEQ ID NO 18: Peptide sequence contained in the amino acid sequence coding for an S.b.-OK1 protein from millet.

SEQ ID NO 19: Peptide sequence contained in the amino acid sequence coding for an S.b.-OK1 protein from millet.

SEQ ID NO 20: Peptide sequence contained in the amino acid sequence coding for an S.b.-OK1 protein from millet.

SEQ ID NO 21: Partial nucleic acid sequence coding for an S.b.-OK1 protein from millet. This nucleic acid sequence has been identified by means of the peptide sequences shown under SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19 and SEQ ID NO 20 using the "Blast Search" facility in the TIGR database.

SEQ ID NO 22: Partial amino acid sequence coding for an S.b.-OK1 protein from millet. The amino acid sequence shown can be derived from the nucleic acid sequence shown under SEQ ID NO 21.

SEQ ID NO 23: Peptide sequence contained in the amino acid sequence coding for a T.a.-OK1 protein from wheat.

SEQ ID NO 24: Peptide sequence containing the amino acid sequence coding for a T.a.-OK1 protein from wheat.

SEQ ID NO 25: Partial nucleic acid sequence coding for a T.a.-OK1 protein from wheat. This nucleic acid sequence has been identified by means of the peptide sequences shown under SEQ ID NO 23 and SEQ ID NO 24 using the "Blast Search" facility in the TIGR database.

SEQ ID NO 26: Partial amino acid sequence coding for a T.a.-OK1 protein from wheat. The amino acid sequence shown can be derived from the nucleic acid sequence shown under SEQ ID NO 25.

Description of the Figures

Fig. 1: Denaturing acrylamide gel for identifying proteins from *Arabidopsis thaliana*, which preferably bind to non-phosphorylated starch in comparison with phosphorylated starch. A standard protein molecular weight marker is shown in trace "M". Proteins obtained after incubating control preparation C from Example 1 d) are shown in trace "-". Protein extracts of *Arabidopsis thaliana*, obtained after incubation with non-phosphorylated starch, isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant (Preparation B, Example 1 d)), are shown in trace "K". Protein extracts of *Arabidopsis thaliana*, obtained after incubation with starch, isolated from leaves of an *Arabidopsis thaliana* sex1-3 which was phosphorylated retrospectively *in vitro* with an R1 protein (Preparation A, Example 1 d), are shown in trace "P". On completion of electrophoresis, the acrylamide gel was stained with Coomassie Blue.

Fig. 2: Demonstration of the autophosphorylating activity of the OK1 protein. Fig. 2A) shows a denaturing (SDS) acrylamide gel stained with Coomassie Blue on completion of the electrophoresis. Figure 2 B) shows the autoradiography of a denaturing (SDS) acylamide gel. The same amounts of the same samples were applied to each of the two gels. M: Standard protein molecular weight marker; R1: Sample from reaction vessel 1 according to Example 7 (after incubating an OK1 protein with ATP); R2: Sample from reaction vessel 2 according to Example 7 (after incubating an OK1 protein with ATP the protein was heated to 95°C); R3: Sample from reaction vessel 3 according to Example 7 (after incubating an OK1 protein with ATP the protein was incubated in 0.5 M HCl); R4: Sample from reaction vessel 4 according to Example 7 (after incubating an OK1 protein with ATP the protein was incubated in 0.5 M NaOH).

Fig. 3: Demonstration of the starch-phosphorylating activity of an OK1 protein (see Example 6). OK1 protein was incubated with non-phosphorylated starch isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant (Preparation A) and starch isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant, which was phosphorylated retrospectively *in vitro* with an R1 protein (Preparation B). Preparation C is the same as Preparation B, except that this Preparation C was incubated without OK1 protein. Two independent tests were carried out for each preparation (A, B, C) (Test 1 and Test 2). The respective amounts are shown, measured in cpm (counts per minute), on ^{33}P labeled phosphate, which was introduced into non-phosphorylated starch (Preparation A) and phosphorylated starch (Preparation B).

Fig. 4: Comparison of the C-atom positions of glucose molecules of the starch, which was phosphorylated from an R1 protein and an OK1 protein respectively (see Example 9). OK1 protein (Preparation A) was incubated in the presence of ATP labeled with ^{33}P with starch isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant, which was phosphorylated retrospectively *in vitro* with an R1

protein.). R1 protein (preparation B) was incubated in the presence of ATP labeled with 33P with starch isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant. After incubation had been completed, a total hydrolysis of the starch was carried out and the hydrolysis products obtained were separated using HPAE chromatography. As standard, glucose-6-phosphate and glucose-3-phosphate were added to the hydrolysis products before separation. The hydrolysis products separated by means of HPAE chromatography were collected in individual fractions. The added glucose-6-phosphate eluted with fraction 15 and the added glucose-3-phosphate with fraction 17. The fractions obtained were subsequently investigated for the presence of radioactively labeled phosphate. The amount of ^{33}P labeled phosphate measured in the individual fractions, measured in cpm (counts per minute), which was introduced into the hydrolysis products of the phosphorylated starch by the OK1 protein or the R1 protein, is shown graphically.

Fig. 5 Demonstration of the autophosphorylation of the OK1 protein. Figure 5 A) shows a Western Blot. Figure 5 B) shows the autoradiography of a denaturing (SDS) acrylamide gel. The same amounts of the same samples were applied to each of the two gels. The OK1 protein was incubated either with randomised radioactively labeled ATP or with ATP specifically radioactively labeled in the gamma position. On completion of incubation, the proteins were either heated to 30°C or 95°C, or incubated in 0.5 M NaOH or 0.5 M HCl respectively.

Fig. 6 Demonstration of the transfer of the beta-phosphate residue of ATP to starch in a reaction catalysed by an OK1 protein. Either ATP specifically labeled with ^{33}P in the gamma position or randomised ^{33}P ATP was used to phosphorylate starch, which had been phosphorylated *in vitro* by means of an R1 protein and isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant, by means of an OK1 protein. No OK1 protein was added in any of the experiments designated as "control". Each preparation was tested twice, independently of one another. The results of both tests are shown.

Fig. 7 Western Blot analysis of protein extracts from plants using an antibody against the OK1 protein from *Arabidopsis thaliana*. Protein extracts from leaves of the following plants are shown: Ara, *Arabidosis thaliana*; 51, 54, 55, 67, 72, 73, 79, 62, 63, 64, 65, 69, 66, 68 are independent lines of the transformation 385JH; D wildtype *Solanum tuberosum* cv Désirée.

General methods

In the following, methods are described, which can be used for carrying out the method according to the invention. These methods constitute specific embodiments of the present invention but do not restrict the present invention to these methods. The person skilled in the art knows that he can implement the invention in the same way by modifying the methods described and/or by replacing individual parts of the methods by alternative parts of the methods.

1. Manufacture of protein extracts from plant tissue

a) Manufacture of protein extracts from plant tissues

Leaf material is frozen in liquid nitrogen immediately after harvesting and subsequently homogenised in the mortar under liquid nitrogen. The reduced leaf material is mixed with ca. 3.5 times the volume (relative to the weight of the leaf material used) of cold (4°C) binding buffer and macerated for 2x10 s using an Ultraturrax (maximum speed). After the first treatment with an Ultraturrax, the reduced leaf material is cooled on ice before the second treatment is carried out. The treated leaf material is then passed through a 100 µm nylon mesh and centrifuged for 20 min (50 ml centrifuge vessel, 20,000xg, 4°C).

b) Precipitation of the proteins contained in the protein extracts

The supernatant obtained following centrifugation according to Step a) is removed and its volume determined. To precipitate proteins, ammonium sulphate is added continuously to the supernatant over a period of 30 minutes while stirring on ice down to a final concentration of 75% (weight/volume). The supernatant is subsequently incubated for a further hour on ice while stirring. The proteins precipitated from the supernatant are pelletised at 20,000xg and 4°C for 10 min and the pellet subsequently absorbed in 5 ml of binding buffer, i.e. the proteins present in the pellet are dissolved.

c) Desalting of the precipitated proteins

The dissolved proteins are desalted using a PD10 column filled with Sephadex G25 (Amersham Bioscience, Freiburg, Prod. No. columns: 17-0851-01, Prod. No. Sephadex G25-M: 17-0033-01) at a temperature of 4°C, i.e. the ammonium sulphate used for the precipitation under step b) is separated from the dissolved proteins. The PD10 column is equilibrated with binding buffer before the proteins dissolved in accordance with Step b) are applied. For this purpose, 5 ml of binding buffer are spread over the column in each case. Subsequently, 2.5 ml of the protein solution obtained in accordance with Step b) are added to each column before proteins are eluted from the column with 3.5 ml binding buffer.

d) Determination of the protein concentration

The protein concentration is determined with a Bradford assay (Biorad, Munich, Prod. No. 500-0006 (Bradford, 1976, Anal. Biochem. 72, 248-254)).

e) Composition of the binding buffer [

Binding buffer:	50 mM	HEPES/NaOH (or KOH), pH 7.2
	1 mM	EDTA
	2 mM	Dithioerythritol (DTE)
	2 mM	Benzamidine

2 mM	ε-aminocaproic acid
0.5 mM	PMSF
0.02 %	Triton X-100

2. Isolation of leaf starch

a) Isolation of starch granules from plant tissues

Leaf material is frozen immediately after harvesting in liquid nitrogen. The leaf material is homogenised in portions in the mortar under liquid nitrogen and absorbed into a total of ca. 2.5-times the volume (weight/volume) of starch buffer. In addition, this suspension is again homogenised in a Waring blender for 20 s at maximum speed. The homogenate is passed through a nylon mesh (100 µm mesh width) and centrifuged for 5 minutes at 1,000xg. The supernatant with the soluble proteins is discarded.

b) Purifying the starch isolated from the plant tissues

After removing the green material lying on top of the starch by rinsing off the green material with starch buffer, the pellet containing the starch obtained from Step a) is absorbed in starch buffer and successively passed through nylon meshes with different mesh widths (in the order 60 µm, 30 µm, 20 µm). The filtrate is centrifuged using a 10 ml Percoll cushion (95% (v/v) Percoll (Pharmacia, Uppsala, Sweden), 5% (v/v) 0.5M HEPES-KOH pH7.2) (Correx tube, 15 min, 2,000xg). The sediment obtained after this centrifugation is resuspended once in starch buffer and centrifuged again (5 min, 1,000xg).

c) Removal of the proteins bound to the starch

Following Step b), starch granules are obtained, which contain proteins bound to the starch. The proteins bound to the surface of the starch granules are removed by incubating four times with 0.5 % SDS (sodium lauryl sulphate) for 10-15 minutes in each case at room temperature under agitation. Each washing step is followed by a

centrifugation (5 min, 5,000xg), in order to separate the starch granules from the respective wash buffer.

d) Purifying the starch that has been freed of proteins

The starch obtained from Step c), which has been freed from the proteins bound to its surface, is subsequently removed by incubating four times with wash buffer for 10-15 minutes in each case at room temperature under agitation. Each washing step is followed by a centrifugation (5 min, 1,000xg), in order to separate the starch granules from the respective wash buffer. These purification steps serve mainly to remove the SDS used in the incubations in Step c).

e) Determination of the concentration of isolated starch

The amount of starch isolated in Step d) is determined photometrically. After suitable dilution, the optical density of the starch suspension is measured against a calibration curve at a wavelength of 600 nm. The linear range of the calibration curve is located between 0 and 0.3 extinction units.

To produce the calibration curves, starch, for example isolated from leaves of an *Arabidopsis thaliana sex1-3* mutant, is dried under vacuum, weighed and absorbed in a defined volume of water. The suspension so obtained is diluted with water in several steps in a ratio of 1 to 1 in each case until a suspension of ca. 5 µg starch per ml of water is obtained. The suspensions obtained by the individual dilution steps are measured in the photometer at a wavelength of 600 nm. The absorption values obtained for each suspension are plotted against the concentration of starch in the respective suspension. The calibration curve obtained should follow a linear mathematical function in the range from 0 µg starch per ml of water to 0.3 µg starch per ml of water.

f) Storage of isolated starch

The starch can either be used directly without further storage for further tests, or stored in aliquots in 1.5 mL Eppendorf vessels at -20°C. Both the frozen starch and the non-stored, freshly isolated starch can be used, if required, for the methods described in the present invention relating to *in vitro* phosphorylation and/or binding test, for example.

g) Composition of buffers used

1x starch buffer: 20 mM HEPES-KOH, pH 8.0
0.2 mM EDTA
0.5 % Triton X-100

Wash buffer: 50 mM HEPES/KOH, pH 7.2

3. Recombinant expression of an identified starch–phosphorylating protein

a) Manufacture of a bacterial expression vector containing a cDNA, which codes for a starch-phosphorylating protein

The cDNA coding for a starch-phosphorylating protein can be amplified, for example, using mRNA or poly-A-plus-mRNA from plant tissues as a "template", by means of a polymerase chain reaction (PCR). For this purpose, a reverse transcriptase is first used for the manufacture of a cDNA strand, which is complementary to an mRNA, which codes for a starch-phosphorylating protein, before the cDNA strand concerned is amplified by means of DNA polymerase. So-called "kits" containing substances, enzymes and instructions for carrying out PCR reactions are available for purchase (e.g. SuperScript™ One-Step RT-PCR System, Invitrogen, Prod. No.: 10928-034. The amplified cDNA coding a starch phosphorylating protein can then be cloned in a bacterial expression vector e.g. pDEST™17(Invitrogen). pDEST™17 contains the T7 promoter which is used to initiate the transcription of the T7-RNA polymerase. Furthermore, the expression vector pDEST™17 contains a Shine Dalgarno sequence in the 5´-direction of the T7 promoter followed by a start codon (ATG) and by a so-

called His tag. This His tag consists of six codons directly following one another, which each code for the amino acid histidine and are located in the reading frame of the said start codon. The cloning of a cDNA coding for a starch-phosphorylating protein in pDEST™17 is carried out in such a way that a translational fusion occurs between the codons for the start codon, the His tag and the cDNA coding for a starch-phosphorylating protein. As a result of this, following transcription initiated on the T7 promoter, and subsequent translation, a starch-phosphorylating protein is obtained, which contains additional amino acids containing the His tag on its N-terminus.

However, other vectors, which are suitable for expression in microorganisms, can also be used for the expression of a starch-phosphorylating protein. Expression vectors and associated expression strains are known to the person skilled in the art and are also available for purchase from the appropriate dealer in suitable combinations.

b) Manufacture of expression clones in *Escherichia coli*

First of all, an appropriate transformation-competent *E. coli* strain, which chromosomally codes for a T7-RNA polymerase, is transformed with the expression plasmid manufactured under Step a), and subsequently incubated overnight at 30°C on culture medium solidified with agar. Suitable expression strains are, for example, BL21 strains (Invitrogen Prod. No.: C6010-03), which chromosomally code for a T7-RNA polymerase under the control of an IPTG-inducible promoter (lacZ).

Bacteria colonies resulting from the transformation can be investigated using methods known to the person skilled in the art to see whether they contain the required expression plasmid containing a cDNA coding for the starch-phosphorylating protein. At the same time, expression clones are obtained.

c) Expression of a starch-phosphorylating protein in *Escherichia coli*

First of all, a preliminary culture is produced. To do this, an expression clone obtained in accordance with Step b) is seeded in 30 ml Terrific Broth (TB medium) containing an antibiotic for selection on the presence of the expression plasmid, and incubated overnight at 30°C under agitation (250 rpm).

A main culture for the expression of a starch-phosphorylating protein is then produced. To do this, in each case, 1 litre Erlenmeyer flasks, each containing 300 ml of TB medium, pre-heated to 30°C, and an antibiotic for selection on the presence of the expression plasmid are each seeded with 10 ml of an appropriate pre-culture and incubated at 30°C under agitation (250 rpm) until an optical density (measured at a wavelength of 600 nm (OD_{600}) of ca. 0.8 is achieved.

If, for the expression of a starch-phosphorylating protein, an expression plasmid is used, in which the expression of the starch-phosphorylating protein is initiated by means of an inducible system (e.g. the expression vector pDEST™17 in BL21 *E. coli* strains, inducible by means of IPTG), then on reaching an OD_{600} of ca. 0.8, the inductor concerned (e.g. IPTG) is added to the main culture. After adding the inductor, the main culture is incubated at 30°C under agitation (250 rpm) until an OD_{600} of ca. 1.8 is achieved. The main culture is then cooled for 30 minutes on ice before the cells of the main culture are separated from the culture medium by centrifugation (10 minutes at 4,000xg and 4°C).

4. Purification of a starch-phosphorylating protein

a) Breaking down of cells expressing a starch-phosphorylating protein

The cells obtained in Step c), Item 3 General Methods are resuspended in lysis buffer. In doing so, ca. 4 ml lysis buffer is added to about 1 g of cells. The resuspended cells are then incubated for 30 minutes on ice before they are broken down using an utrasonic probe (Baudelin Sonoplus UW 2070, Baudelin electronic, Berlin, settings: Cycle 6, 70%, 1 minute) under continuous cooling by means of the ice. Care must be taken here to ensure that the cell suspension is not heated too much during the ultrasonic treatment. The suspension obtained after the ultrasonic

treatment is centrifuged (12 minutes at 20,000xg, 4°C) and the supernatant obtained after centrifugation is filtered using a filter with a pore size of 45 µm.

b) Purification of the starch-phosphorylating protein

If the starch-phosphorylating protein expressed in *E. coli* cells is a fusion protein with a His tag, then purification can take place using nickel ions, to which the His tag binds with greater affinity. To do this, 25 ml of the filtrate obtained in Step d) is mixed with 1 ml Ni-agarose slurry (Qiagen, Prod. No.: 30210) and incubated for 1 hour on ice. The mixture of Ni-agarose slurry and filtrate is subsequently spread over a polystyrene column (Pierce, Prod. No.: 29920). The product, which runs through the column, is discarded. The column is next washed by adding 8 ml of lysis buffer, the product, which runs through the column, again being discarded. Elution of the starch-phosphorylating protein then takes place by fractionated addition to the column of 1 ml E1 buffer twice, followed by 1 ml E2 buffer once and subsequently 1 ml E3 buffer five times. The product, which runs through the column, which is produced by adding the individual fraction of the appropriate elution buffer (E1, E2, E3 buffer) to the column, is collected in separate fractions. Aliquots of these fractions are subsequently analysed by means of denaturing SDS acrylamide gel electrophoresis followed by Coomassie Blue staining. The fractions, which contain the starch-phosphorylating protein in sufficient quantity and satisfactory purity, are purified and concentrated using pressurised filtration at 4°C. Pressurised filtration can be carried out, for example, using an Amicon cell (Amicon Ultrafiltration Cell, Model 8010, Prod. No.: 5121) using a Diaflo PM30 membrane (Millipore, Prod. No.: 13212) at 4°C. Other methods known to the person skilled in the art can also be used for concentration however.

c) Composition of buffers used

Lysis buffer:	50 mM	HEPES	
	300 mM	NaCl	
	10 mM	Imidazole	

pH 8.0 (adjust with NaOH)

1 mg/ml Lysozyme (add immediately before using the buffer)

¼ tablet per 10 ml of protease inhibitors Complete EDTA free, (Roche Product No.: 1873580) (add immediately before using the buffer)

Elution buffer E1: 50 mM HEPES
300 mM NaCl
50 mM Imidazole
pH 8.0 (adjust with NaOH)

Elution buffer E2: 50 mM HEPES
300 mM NaCl
75 mM Imidazole
pH 8.0 (adjust with NaOH)

Elution buffer E3: 50 mM HEPES
300 mM NaCl
250 mM Imidazole
pH 8.0 (adjust with NaOH)

5. Recombinant expression of an R1 protein

The recombinant expression of an R1 protein is described in the literature (Ritte et al., 2002, PNAS 99, 7166-7171; Mikkelsen et al., 2004, Biochemical Journal 377, 525-532), but can also be carried out in accordance with the methods relating to the

recombinant expression of a starch-phosphorylating protein described above under Item 3, General Methods.

6. Purification of an R1 protein

The purification of an R1 protein is described in the literature (Ritte et al., 2002, PNAS 99, 7166-7171; Mikkelsen et al., Mikkelsen et al., 2004, Biochemical Journal 377, 525-532), but can also be carried out in accordance with the methods relating to the purification of a starch-phosphorylating protein described above under Item 4, General Methods if an R1 fusion protein, which contains a His tag, is produced by expression of R1 in *E. coli* cells.

7. In vitro manufacture of phosphorylated starch starting from non-phosphorylated starch

a) *In vitro* phosphorylation of non-phosphorylated starch

Starch, which does not contain starch phosphate (e.g. isolated from leaves of *Arabidopsis thaliana sex1-3* mutants using the methods described above under Item 2, General Methods), is mixed with R1 buffer and with purified R1 protein (ca. 0.25 µg R1 protein per mg starch) in order to produce a starch content of 25 mg per ml. This reaction preparation is incubated overnight (ca. 15 h) at room temperature under agitation. R1 bound to the starch present in the reaction preparation is removed on completion of the reaction by washing four times with ca. 800 µl 0.5 % SDS in each case. Subsequently, the SDS still present in the *in vitro* phosphorylated starch is removed by washing five times with 1 ml wash buffer in each case. All washing steps are carried out at room temperature for 10 to 15 minutes under agitation. Each washing step is followed by a centrifugation (2 min, 10,000xg), in order to separate the starch granules from the respective SDS buffer or wash buffer.

b) Composition of buffers used

R1 buffer: 50 mM HEPES/KOH, pH 7,5
1 mM EDTA
6 mM $MgCl_2$
0.5 mM ATP

Wash buffer: 50 mM HEPES/KOH, pH 7.2

8. Binding of proteins to phosphorylated starch or non-phosphorylated starch

a) Isolation of P-starch protein complexes or non-phosphorylated starch protein complexes

Ca. 50 mg of P-starch or ca. 50 mg of non-phosphorylated starch are resuspended in separate preparations in ca. 800 µl of protein extract in each case. The protein concentration of the protein extracts should be ca. 4 mg to 5 mg per ml in each case. The incubation of the P-starch or non-phosphorylated starch with protein extracts is carried out at room temperature for 15 minutes at 4°C under agitation. On completion of the incubation, the reaction preparations are centrifuged out using a Percoll cushion (4 ml) (15 minutes, 3500 rpm, 4°C). After centrifugation, proteins that are not bound to phosphorylated starch or P-starch will be found in the supernatant and can be removed with a Pasteur pipette. The supernatant is discarded. The sedimented pellet containing P-starch and non-phosphorylated starch, including the proteins bound to the respective starches (P-starch protein complexes or non-phosphorylated starch protein complexes respectively), obtained after centrifugation is washed twice with 1 ml of wash buffer in each case (see above, General Methods under item 7.b) by incubating for 3 minutes at 4°C in each case under agitation. The washing step is followed by a centrifugation (5 minutes, 8000 rpm, 4°C in a table centrifuge, Hettich EBA 12R) in order to separate the P-starch or non-phosphorylated starch respectively from the wash buffer.

b) Dissolving the proteins bound in the P-starch protein complexes or non-phosphorylated starch protein complexes respectively

The P-starch protein complexes or non-phosphorylated starch protein complexes respectively obtained in Step a) are resuspended in ca. 150 µl SDS test buffer and incubated at room temperature for 15 minutes under agitation. The P-starch or non-phosphorylated starch respectively is subsequently removed from the dissolved proteins by centrifugation (1 minute, 13,000 rpm, room temperature, Eppendorf table centrifuge). The supernatant obtained after centrifugation is centrifuged again in order to remove any residues of P-starch or non-phosphorylated starch respectively (1 minute, 13,000 rpm, room temperature, Eppendorf table centrifuge) and removed. As a result, dissolved proteins, which bind to the P-starch or non-phosphorylated starch respectively, are obtained.

c) Composition of buffers used

SDS test buffer:	187.5 mM	Tris/HCl pH 6.8	
	6 %	SDS	
	30 %	Glycerine	
	~ 0.015 %	Bromophenol blue	
	60 mM	DTE (add fresh)	

Percoll: Percoll is dialysed overnight against a solution consisting of and 25 mM HEPES / KOH, pH 7.0

9. Separation of proteins, which bind to P-starch and/or non-phosphorylated starch

The dissolved proteins obtained in Step c) under Item 8. General Methods relating to the binding of proteins to P-starch or non-phosphorylated starch respectively are incubated for 5 minutes at 95°C in each case and subsequently separated using

denaturing polyacrylamide gel electrophoresis. In doing so, an equal volume is applied to the acrylamide gel in each case for the dissolved proteins obtained by binding to P-starch and for those obtained by binding to non-phosphorylated starch. The gel obtained on completion of electrophoresis is stained at least overnight with colloidal Coomassie (Roth, Karlsruhe, Roti-Blue Rod. No.: A152.1) and subsequently decolourised in 30 % methanol, 5 % acetic acid, or in 25% methanol.

10. Identification and isolation of proteins, which bind to P-starch and/or non-phosphorylated starch

a) Identification of proteins with increased binding activity towards P-starch in comparison with non-phosphorylated starch

Proteins, which, after separation by means of acrylamide gel electrophoresis and subsequent visualisation by staining (see above, Item 9, General Methods), exhibit an increased signal after binding to P-starch in comparison with a corresponding signal after binding to non-phosphorylated starch, have increased bonding activity towards P-starch in comparison with non-phosphorylated starch. By this means, it is possible to identify proteins, which have increased binding activity towards P-starch in comparison with non-phosphorylated starch. Proteins, which have increased binding activity towards P-starch in comparison with non-phosphorylated starch, are excised from the acrylamide gel.

b) Identification of proteins, which have increased binding activity towards P-starch in comparison with non-phosphorylated starch

Proteins identified in accordance with Step a) are digested with trypsin and the peptides obtained are analysed by means of MALDI-TOF to determine the masses of the peptides obtained. Trypsin is a sequence-specific protease, i.e. trypsin only splits proteins at a specified position when the proteins concerned contain certain amino acid sequences. Trypsin always splits peptide bonds when the amino acids arginine and lysine follow one another starting from the N-terminus. In this way, it is possible to theoretically determine all peptides that would be produced following the trypsin

digestion of an amino acid sequence. From the knowledge of the amino acids coding for the theoretically determined peptides, the masses of the peptides, which are obtained after theoretical trypsin digestion, can also be determined. Databases (e.g. NCBInr http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm; Swissprot http://cbrg.inf.ethz.ch/Server/MassSearch.html), which contain information concerning the masses of peptides after theoretical trypsin digestion, can therefore be compared with the real masses of peptides of unknown proteins obtained with MALDI-TOF-MS. Amino acid sequences, which have the same peptide masses after theoretical and/or real trypsin digestion, are to be looked upon as being identical. The databases concerned contain both peptide masses of proteins, the function of which has already been shown, and also peptide masses of proteins, which up to now only exist hypothetically by derivation from amino acid sequences starting from nucleic acid sequences obtained in sequencing projects. The actual existence and the function of such hypothetical proteins has therefore seldom been shown and, if there is a function at all, then this is usually based only on predictions and not on an actual demonstration of the function.

Bands containing proteins obtained in accordance with Step a) are excised from the acrylamide gel; the excised acrylamide piece is reduced and decolourised by incubating for approximately half an hour at 37°C in ca. 1 ml 60% 50mM NH_4HCO_3, 40% acetonitrile. The decolourising solution is subsequently removed and the remaining gel dried under vacuum (e.g. Speedvac). After drying, trypsin solution is added to digest the proteins contained in the gel piece concerned. Digestion takes place overnight at 37°C. After digestion, a little acetonitrile is added (until the acrylamide gel is stained white) and the preparation dried under vacuum (e.g. Speedvac). When drying is complete, just enough 5% formic acid is added to cover the dried constituents and incubated for a few minutes at 37°C. The acetonitrile treatment followed by drying is repeated once more. The dried constituents are subsequently absorbed in 0.1% TFA (trifluoroacetic acid, 5 µl to 10 µl) and dripped onto a carrier in ca. 0.5 µl portions. Equal amounts of matrix (ε-Cyano-4-hydroxy-cinnamic acid) are also applied to the carrier After crystallising out the matrix, the masses of peptides are determined by means of MALDI-TOF-MS-MS (e.g. Burker

ReflexTM II, Bruker Daltonic, Bremen) With the masses obtained, databases are searched for amino acid sequences, which give the same masses after theoretical trypsin digestion. In this way, amino acid sequences can be identified, which code for proteins, which preferably bind to phosphorylated alpha-1,4-glucans and/or which need P-alpha-1,4-glucans as a substrate.

11. Method for demonstrating starch-phosphorylating activity of a protein

a) Incubation of proteins with P-starch and/or non-phosphorylated starch

In order to demonstrate whether a protein has starch-phosphorylating activity, proteins to be investigated can be incubated with starch and radioactively labeled ATP. To do this, ca. 5 mg of P-starch or ca. 5 mg of non-phosphorylated starch are incubated with the protein to be investigated (0.01 µg to 5.0 µg per mg of starch used) in 500 µl phosphorylation buffer for 10 minutes to 30 minutes at room temperature under agitation. The reaction is subsequently stopped by the addition of SDS up to a concentration of 2% (weight/volume). The starch granules in the respective reaction mixture are centrifuged out (1 minute, 13,000xg), and washed once with 900 µl of a 2 % SDS solution and four times each with 900 µl of a 2 mM ATP solution. Each washing step is carried out for 15 minutes at room temperature under agitation. After each washing step, the starch granules are separated from the respective wash buffer by centrifugation (1 min, 13,000xg).

In addition, when carrying out an experiment to demonstrate starch-phosphorylating activity of a protein, further reaction preparations, which do not contain protein or contain inactivated protein, but which are otherwise treated in the same way as the reaction preparations described, should be processed as so-called controls.

b) Determination of the amount of phosphate residues incorporated in the P-starch and/or non-phosphorylated starch due to enzymatic activity

The starch granules obtained in accordance with Step a) can be investigated for the presence of radioactively labeled phosphate residues. To do this, the respective starch is resuspended in 100 µl of water and mixed with 3 ml of scintillation cocktail in

each case (e.g. Ready Safe™, BECKMANN Coulter) and subsequently analysed using a scintillation counter (e.g. LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER™).

c) Identification of proteins, which preferably use P-starch as a substrate

If a protein is incubated in separate preparations, once with P-starch and once with non-phosphorylated starch, in accordance with the method described under a), then, by comparing the values for the presence of starch phosphate obtained according to Step b), it can be determined whether the protein concerned has incorporated more phosphate in P-starch in comparison with non-phosphorylated starch. Thus, proteins which can introduce phosphate into P-starch but not into non-phosphorylated starch can also be identified, i.e., proteins which already require phosphorylated starch as substrate for a further phosphorylation reaction can be identified.

d) Composition of buffers used

Phosphorylation buffer:	50 mM	HEPES/KOH, pH 7.5
	1 mM	EDTA
	6 mM	$MgCl_2$
	0.01 to 0.5 mM	ATP

0.2 to 2 µCi per ml randomised ^{33}P-ATP (alternatively, ATP, which contains a phosphate residue, which is specifically labeled in the gamma position, can also be used)

In conjunction with the present invention, the term "randomised ATP" is to be understood to mean ATP, which contains labeled phosphate residues both in the gamma position and in the beta position (Ritte et al. 2002, PNAS 99, 7166-7171). Randomised ATP is also described in the scientific literature as beta/gamma ATP. A method for manufacturing randomised ATP is described in the following.

i) Manufacture of randomised ATP

The method described here for manufacturing randomised ATP using enzyme catalysed reactions is based on the following reaction mechanisms:

1. reaction step 1

γ^{33}P-ATP + AMP + myokinase → β^{33}P-ADP + ADP

(Adenosine-P-P-^{33}P + Adenosine-P → Adenosine-P-P + Adenosine-P-^{33}P)

2. reaction step 2

^{33}P-ADP + ADP + 2 PEP + Pyruvate kinase → β^{33}P-ATP + ATP + 2 Pyruvate

(Adenosine-P-P + Adenosine-P-^{33}P + 2 PEP → Adenosine-P-P-P + Adenosine-P-^{33}P-P + 2 Pyruvate)

The reaction equilibria lie on the product side but, in spite of this, this reaction produces a mixture consisting mainly of β^{33}P-ATP and some γ^{33}P-ATP.

ii) Carrying out the 1st reaction step

ATP (100 µCi, 3000 Ci per mmol), which contains a phosphate residue labeled with ^{33}P in the gamma position (Hartmann Analytic, 10 µCi/µl), is incubated with 2 µl myokinase (AMP-phosphotransferase, from rabbit muscle; SIGMA, Prod. No.: M3003 3.8 mg/ml, 1,626 units/mg) in 90 µl randomising buffer for 1 hour at 37°C. The reaction is subsequently stopped by incubating for 12 minutes at 95°C before the reaction preparation is purified by means of centrifugal filtration using a Microcon YM 10 filter (Amicon, Millipore Prod. No. 42407) at 14,000xg for at least 10 minutes.

iii) Carrying out the 2nd reaction step

2 µl pyruvate kinase (see below for how to manufacture an appropriate solution) and 3 µl 50 mM PEP (phosphoenolpyruvate) are added to the filtrate obtained in Step ii). This reaction mixture is incubated for 45 minutes at 30°C before the reaction is stopped by incubating at 95°C for 12 minutes. The reaction mixture is subsequently centrifuged (2 minutes, 12,000 rpm in an Eppendorf table centrifuge). The

supernatant containing randomised ATP obtained after centrifugation is removed, aliquoted and can be stored at -20°C.

Manufacture of the pyruvate kinase solution

15 µl pyruvate kinase (from rabbit muscle, Roche, Prod. No. 12815, 10 mg/ml, 200 units/mg at 25 °C) are centrifuged out, the supernatant discarded and the pellet absorbed in 27 µl pyruvate kinase buffer.

iv) Buffers used

Pyruvate kinase buffer:	50 mM	HEPES/KOH	pH 7.5
	1 mM	EDTA	
Randomising buffer:	100 mM	HEPES/KOH	pH 7.5
	1 mM	EDTA	
	10 %	Glycerol	
	5 mM	$MgCl_2$	
	5 mM	KCl	
	0.1 mM	ATP	
	0.3 mM	AMP	

12. Demonstration of the autophosphorylation of a protein

In order to demonstrate whether a protein has autophosphorylating activity, proteins to be investigated can be incubated with radioactively labeled ATP. To do this, proteins to be investigated (50 µg to 100 µg) are incubated in 220 µl phosphorylation buffer (see above, Item 12 d), General Methods) for 30 minutes to 90 minutes at room temperature under agitation. The reaction is then stopped by adding EDTA up to a final concentration of 0.11 M. Ca. 2 µg to 4 µg of protein is then separated using denaturing polyacrylamide electrophoresis (7.5% acrylamide gel). The gel obtained

after polyacrylamide gel electrophoresis is subjected to autoradiography. Proteins, which exhibit a signal in the autoradiography, carry a radioactive phosphate residue.

13. Identification of the C-atom positions of the glucose molecules of an alpha-1,4-glucan, into which phosphate residues are introduced by a starch-phosphorylating protein

Which C-atom positions of the glucose molecules of an alpha-1,4-glucan are phosphorylated by a protein can be demonstrated in a controlled manner by hydrolysis of the phosphorylated glucans obtained by means of an appropriate protein *in vitro*, subsequent separation of the glucose monomers obtained after hydrolysis, followed by measurement of the phosphate incorporated by an appropriate protein in certain fractions of the glucose molecules.

a) Total hydrolysis of the alpha-1,4-glucans

Water suspensions containing alpha-1,4-glucan are centrifuged, the sedimented pellet subsequently resuspended in 0.7 M HCl (Baker, for analysis) and incubated for 2 hours at 95°C under agitation. On completion of incubation, the samples are briefly cooled and centrifuged (e.g. 2 minutes 10,000xg). The supernatant obtained is transferred to a new reaction vessel and neutralised by the addition of 2 M NaOH (Baker, for analysis). If a pellet remains, it is resuspended in 100 µl of water and the quantity of labeled phosphate present therein is determined as a control.

The neutralised supernatant is subsequently centrifuged over a 10 kDa filter. By measuring an aliquot of the filtrate obtained, the quantity of labeled phosphate in the filtrate is determined using a scintillation counter, for example.

b) Fractionation of the hydrolysis products and determination of the phosphorylated C-atom positions

The neutralised filtrates of the hydrolysis products obtained by means of Step a) can be separated (when using radioactively labeled ATP about 3000 cpm) using high-pressure anion exchange chromatography (HPAE), for example. The neutralised filtrate can be diluted with H_2O to obtain the volume required for HPAE. In addition, glucose-6-phosphate (ca. 0.15 mM) and glucose-3-phosphate (ca. 0.3 mM) are added to the appropriate filtrates in each case as an internal control. Separation by means of HPAE can be carried out, for example, using a Dionex DX 600 Bio Lc system using a CarboPac PA 100 column (with appropriate pre-column) and a pulsed amperometric detector (ED 50). In doing so, before injecting the sample, the column is first rinsed for 10 minutes with 99% eluent C and 1% eluent D. A sample volume of 60 µl is then injected. The elution of the sample takes place under the following conditions:

Flow rate: 1 ml per minute

Gradient: linearly increasing from 0 minutes to 30 minutes

	Eluent C	Eluent D
0 minutes	99%	1%
30 minutes	0%	100%
35 Minutes	0%	100%
Run terminated		

The hydrolysis products eluted from the column are collected in individual fractions of 1 ml each. As, in each case, non-labeled glucose-3-phosphate (Ritte et al. 2002, PNAS 99, 7166-7171) and non-labeled glucose-6-phosphate (Sigma, Prod. No.: G7879) have been added to the injected samples of hydrolysis products as internal standards, the fractions, which contain either glucose-3-phosphate or glucose-6-phosphate, can be determined by means of pulsed amperometric detection. By measuring the amount of labeled phosphates in the individual fractions and subsequently comparing with the fractions, which contain glucose-3-phosphate or glucose-6-phosphate, this can be used to determine those fractions, containing labeled glucose-6-phosphate or labeled glucose-3-phosphate. The amount of labeled

phosphate in the fraction concerned is determined. From the ratios of the amounts of glucose-3-phosphate to glucose-6-phosphate measured for labeled phosphate in the individual hydrolysis products, it can now be determined which C-atom position is preferably phosphorylated by an alpha-1,4-glucan phosphorylating enzyme.

c) Buffers used

Eluent C: 100 mM NaOH

Eluent D: 100 mM NaOH

500 mM sodium acetate

14. Preparation of the samples for the sequencing using Q-TOF-MS-MS

a) General remarks

Isolated proteins which can also be present in the form of bands excised from polyacrylamide gels, are first cleaved into smaller fragments by means of a trypsin digestion. The peptides formed are introduced into a hybrid mass spectrometer in which a time-of-flight (TOF) mass spectrometer is coupled to a quadrupole mass spectrometer. In the first phase of the measurement the first mass spectrometer (the quadrupole) is "switched off" and the masses of the peptides formed in the digestion can be determined in the TOF mass spectrometer. In the second phase a selected peptide is "filtered out" in the quadrupole, i.e., only this peptide can pass the quadrupole, all the others are deflected. The peptide is then broken by colliding with charged gas molecules in the "collision cell". In this case the "breaks" occur mainly at the peptide bonds. As a result, more or less statistically distributed peptide fragments which differ in mass are formed. The amino acid sequence of the peptides can then be determined by "sorting" these fragments. If overlapping peptides are obtained, the amino acid sequence of a protein can thus be obtained. The use of mass spectroscopy for identification and sequencing is known to the person skilled in the art and is sufficiently described in the specialist literature [e.g. P. Michael Conn (Ed.),

2003, Humana Press, New Jersey, ISBN: 1-58829-340-8]; J.R. Chapman (Ed.), 2000, Humana Press, SBN: 089603609X].

b) Reduction and alkylation of cysteine residues of proteins

The cysteine residues containing the amino acid sequences of the proteins to be analysed can be reduced/alkylated by means of gel electrophoresis before separation of the proteins. For this purpose, the proteins which are to be separated by means of gel electrophoresis are mixed with SDS sample buffer (must not contain any DTT or beta-mercaptoethanol). Freshly prepared DTT is then added to these samples up to a final concentration of 10 mM and the sample incubated for 3 minutes at 95°C. After cooling the sample to room temperature, freshly prepared iodacetamide is added up to a final concentration of 20 mM. The sample is incubated for 20 minutes at room temperature in the dark. The proteins present in the samples are then separated by means of acrylamide gel electrophoresis.

c) Isolation of the proteins from the acrylamide gel

Protein bands containing proteins whose sequences are to be determined are excised using a clean scalpel as "edgeless" as possible and reduced (ca. 1 mm^3-cube). The reduced gel pieces are placed in a 0.5 ml or 1.5 ml reaction vessel and sedimented by short centrifugation.

d) Decolourisation of the excised gel pieces

If gels stained using silver ions were used, the gel pieces obtained according to step c) are completely covered with a solution containing 30 mM K-ferricyanide and 100 mM Na-thiosulphate in the ratio 1:1 and agitated (Vortex) until the gel pieces are completely decolourised. The decolourising solution is then removed and the gel pieces are washed three times with 200 µl of high-purity water in each case (conductivity ca. 18 MOhm).

If gels stained with Coomassie Blue were used, the gel pieces obtained according to step c) are incubated with a solution containing high-purity water and acetonitrile (degree of purity: at least HPLC pure) in the ratio 1:1 twice for 15 minutes in each case under agitation. The volume of the decolourising solution should correspond to ca. twice the volume of the gel. The washing solution is removed after each washing step.

After decolourisation has been completed, the gel pieces are mixed with one volume (relative to the gel pieces) of acetonitrile and incubated for 15 minutes at room temperature under agitation. The acetonitrile is removed and the gel pieces mixed with one volume of 100 mM ammonium bicarbonate, mixed and incubated for 5 minutes at room temperature. Acetonitrile is then added so as to give a ratio of 1:1 relative to the quantity of ammonium bicarbonate and acetonitrile. Incubation is carried out for a further 15 minutes at room temperature before the solution is removed and the remaining gel pieces are dried under vacuum (e.g. Speedvac).

e) Trypsin digestion of the proteins in the gel pieces

Trypsin solution (10 ng of trypsin per µl of 50 mM ammonium bicarbonate) is added in 10 µl portions to the dry gel pieces obtained according to step d). After every addition of trypsin solution, incubation on ice is carried out for 10 minutes in each case. Trypsin solution is added in portions until the gel pieces do not swell any further and are completely covered by trypsin solution. The trypsin solution is then removed and the gel pieces are incubated overnight at 37°C.

f) Isolation of the peptides from the acrylamide gel

The samples obtained according to step e) are briefly centrifuged in order to collect the liquid contained in the reaction vessel, the liquid is removed and transferred to a new reaction vessel. The gel pieces are treated for 2 minutes with ultrasound (ultrasound water bath). The remaining gel pieces are then mixed with once their volume of 25 mM ammonium bicarbonate solution and incubated for 20 minutes

under agitation. Acetonitrile is then added so that a ratio of ammonium bicarbonate to acetonitrile of 1:1 is adjusted and incubation is carried out at room temperature for a further 15 minutes under agitation. After incubation has been completed, the samples are treated with ultrasound again for 2 minutes before the liquid is removed and combined with the liquid which had been removed previously. The remaining gel pieces are mixed with once their volume of a solution containing 5% formic acid and acetonitrile in the ratio 1:1 and incubated for 15 minutes at room temperature under agitation. The liquid is removed and combined with the liquid which had been removed previously. The incubation of the gel pieces in 5% formic acid / acetonitrile (ratio 1:1) is repeated and the liquid obtained is likewise added to the previously collected liquids. The combined supernatants contain the peptides to be sequenced and are concentrated to ca. 15 µl in the vacuum centrifuge (Speedvac) at 60°C The peptides thus obtained can be stored at 20°C until they are analysed using Q-TOF. Before the proteins can be sequenced in the mass analysis, they can be desalted using methods known to the person skilled in the art.

15. Transformation of rice plants

Rice plants were transformed in accordance with the methods described by Hiei et al. (1994, Plant Journal 6(2), 271-282).

16. Transformation of potato plants

Potato plants were transformed using agrobacterium as described by Rocha-Sosa et al. (EMBO J. 8, (1989), 23-29).

17. Determination of the starch phosphate content

Determination of the C-6 phosphate content

In the starch the positions C2, C3 and C6 of the glucose units can be phosphorylated. 50 mg of starch was hydrolysed in 500 µl of 0.7 M HCl for 4 h at

95°C to determine the C6-P content of the starch. The preparations were then centrifuged for 10 min at 15,500 g and the supernatants removed. From the supernatants 7 µl is mixed with 193 µl of imidazole buffer (100 mM imidazole, pH 7.4; 5 mM $MgCl_2$, 1 mM EDTA and 0.4 mM NAD). The measurement was made using a photometer at 340 mm. After a base absorption had been established, the enzyme reaction was started by adding 2 units of glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Boehringer Mannheim). The change in absorption is directly proportional to the concentration of the G-6-P content of the starch.

b) Determination of the total phosphate content

The total phosphate content was determined using the Ames method (Methods in Enzymology VIII, (1966), 115-118).

Approximately 50 mg of starch is mixed with 30 µl of ethanol magnesium nitrate solution and ashed for three hours at 500°C in a muffle furnace. The residue is mixed with 300 µl of 0.5 M hydrochloric acid and incubated for 30 min at 60°C. An aliquot is then made up to 300 µl of 0.5 M hydrochloric acid, added to a mixture of 100 µl of 10% ascorbic acid and 600 µl of 0.42% ammonium molybdate in 2 M sulphuric acid and incubated for 20 min at 45°C.

c) Determination of the content of C-6 phosphate and C-3 phosphate

To determine the content of phosphate bound in the C-6 position and in the C-3 position of the glucose molecules of an alpha-1,4-glucan, the glucans concerned can be separated using HPAE after total hydrolysis using the method specified under General Methods Item 13. The quantities of glucose-6-phosphate and glucose-3-phosphate can be determined by integrating the individual peak areas obtained after HPEA separation. The quantity of glucose-6-phosphate and glucose-3-phosphate in the samples to be studied can be determined by comparing the peak area for glucose-6-phosphate and glucose-3-phosphate obtained in the unknown samples, with the peak areas obtained after separation using HPAE with known quantities of glucose-6-phosphate and glucose-3-phosphate.

Examples

1. Isolation of a protein from *Arabidopsis thaliana*, which has increased binding activity with respect to P-starch in comparison with non-phosphorylated starch

a) Manufacture of protein extracts from *Arabidopsis thaliana*

Protein extracts were manufactured from approximately 7 g of leaves (fresh weight) of *Arabidopsis thaliana* (Ökotyp Columbia, Col-O) in accordance with the method described under Item 1, General Methods.

b) Isolation of starch granules from leaves of *sex1-3* mutants of *Arabidopsis thaliana*

Starch granules were isolated from approximately 20 g (fresh weight) of leaves of a *sex1-3* mutant of *Arabidopsis thaliana* in accordance with the method described under Item 2, General Methods.

c) *In vitro* phosphorylation of starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana* with purified R1 protein.

About 30 mg of non-phosphorylated starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana* was phosphorylated in accordance with the method described under Item 7, General Methods, by means of an R1 protein recombinantly expressed in *E. coli* and purified. The methods described in Ritte et al. (2002, PNAS 99, 7166-7171) were used for the expression of the R1 protein in *E. coli* and for the subsequent purification.

d) Isolation of proteins, which bind to P-starch and/or non-phosphorylated starch

Protein extracts of *Arabidopsis thaliana*, obtained in accordance with Step a), were incubated and washed in a Preparation A with 50 mg of the *in vitro* phosphorylated starch manufactured in accordance with Step c) using the method described under Item 8 a), General Methods.

In a second Preparation B, protein extracts of *Arabidopsis thaliana*, obtained in accordance with Step a), were incubated and washed with 50 mg of the non-phosphorylated starch manufactured in accordance with Step b) using the method described under Item 8 a), General Methods.

Subsequently, the proteins bound to the P-starch of Preparation A and to the non-phosphorylated starch of Preparation B were dissolved in accordance with the method described under Item 8 b), General Methods.

In a third Preparation C, 50 mg of the *in vitro* phosphorylated starch manufactured in accordance with Step c) were incubated and washed using the method described under Item 8 a), General Methods. Preparation C contained no protein extracts however.

e) Separation of the proteins obtained in accordance with Step d) by means of acrylamide gel electrophoresis

The proteins of Preparations A, B and C obtained in Step d) were separated by means of a 9% acrylamide gel under denaturing conditions (SDS) using the method described under Item 9, General Methods, and subsequently stained with Coomassie Blue. The stained gel is shown in Fig. 1. It can be clearly seen that a protein, which has a molecular weight of ca. 130 kDa in denaturing acrylamide gel referred to a protein standard marker (Trace M), preferably binds to phosphorylated starch (Trace P) in comparison with non-phosphorylated starch (K).

f) Identification of the protein, which preferably binds to P-starch in comparison with non-phosphorylated starch

The band of the protein with a molecular weight of ca. 130 kDa identified in Step e) was excised from the gel. The protein was subsequently released from the acrylamide as described under General Methods 10 b), digested with trypsin and the peptide masses obtained determined by means of MALD-TOF-MS. The so-called "fingerprint" obtained by MALDI-TOF-MS was compared with fingerprints of theoretically digested amino acid molecules in databases (Mascot: http://www.matrixscience.com/search_form_select.html; ProFound: http://129.85.19.192/profound_bin/WebProFound.exe; PepSea: http://195.41.108.38/PepSeaIntro.html). As such a fingerprint is very specific to a protein, it was possible to identify an amino acid molecule. Using the sequence of this amino acid molecule, it was possible to isolate a nucleic acid sequence from *Arabidopsis thaliana* coding for an OK1 protein. The protein identified using this method was designated as A.t.-OK1. After analysing the amino acid sequence from *Arabidopsis thaliana*, it was found that this deviates from the sequence present in the database (NP 198009, NCBI). The amino acid sequence shown in SEQ ID No 2 codes for the A.t.-OK1 protein. SEQ ID No 2 contains deviations when compared with the sequence in the database (Acc.: NP 198009.1, NCBI). The amino acids 519 to 523 (WRLCE) and 762 to 766 (VRARQ) contained in SEQ ID No 2 are not in the sequence, which is present in the database (ACC.: NP 198009.1). NP 198009.1). Compared to version 2 of the database sequence (Acc.: NP 198009.2) the amino acid sequence shown in SEQ ID NO 2 contains the additional amino acids 519 to 523 (WRLCE).

2. Cloning of a cDNA, which codes for the identified OK1 protein

The A.t.-OK1 cDNA was isolated using reverse PCR using mRNA isolated from leaves of *Arabidopsis thaliana*. To do this, a cDNA Strand was synthesised by means of reverse transcriptase (SuperScript™ First-Strand Synthesis System for RT PCR, Invitrogen Prod. No.: 11904-018), which was then amplified using DNA polymerase (Expand High Fidelity PCR Systems, Roche Prod. No.: 1732641). The amplified product obtained from this PCR reaction was cloned into the vector pGEM®(-T

(Invitrogen Prod. No.: A3600). The plasmid obtained is designated A.t.-OK1-pGEM®-T, the cDNA sequence coding for the A.t.-OK1 protein was determined and is shown under SEQ ID NO. 1.®

The sequence shown under SEQ ID NO 1 is not the same as the sequence, which is contained in the database. This has already been discussed for the amino acid sequence coding for an A.t.-OK1 protein.

Conditions used for the amplification of the cDNA coding for the A.t.-OK1 protein

First strand synthesis:

The conditions and buffer specified by the manufacturer were used. In addition, the reaction preparation for the first strand synthesis contained the following substances:

3 µg	Total RNA
5 µM	3'-primer (OK1rev1:5´-GACTCAACCACATAACACACAAAGATC)
0.83 µM	dNTP Mix

The reaction preparation was incubated for 5 minutes at 75°C and subsequently cooled to room temperature.

The 1st strand buffer, RNase inhibitor and DTT were then added and incubated for 2 minutes at 42°C before 1 µL Superscript RT DNA polymerase was added and the reaction preparation incubated for 50 minutes at 42°C.

Conditions for the amplification of the first strand by means of PCR:

1 µL	of the reaction preparation of the first strand synthesis
0.25 µM	3'Primer (OK1rev2:5´- TGGTAACGAGGCAAATGCAGA)
0.25 µM	5'Primer (OK1fwd2:5´-ATCTCTTATCACACCACCTCCAATG)

Reaction conditions:

Step 1	95°C	2 min

Step 2	94°C	20 sec
Step 3	62°C	30 sec
Step 4	68°C	4 minutes
Step 5	94°C	20 sec
Step 6	56°C	30 sec
Step 7	68°C	4 minutes
Step 8	68°C	10 minutes

The reaction was first carried out in accordance with Steps 1 to 4. Ten repeats (cycles) were carried out between Step 4 and Step 2, the temperature of Step 3 being reduced by 0.67°C after each cycle. This was subsequently followed by the reaction in accordance with the conditions specified in Steps 5 to 8. Twenty five repeats (cycles) were carried out between Step 7 and Step 5, the time of Step 7 being increased by 5 sec on each cycle. On completion of the reaction, the reaction was cooled to 4°C.

3. Manufacture of a vector for the recombinant expression of cDNA of the OK1 protein

Following amplification by means of PCR by using the plasmid A.t.-OK1-pGEM® as a template using Gateway Technology (Invitrogen), the sequence coding the OK1 protein from *Arabidopsis thaliana* was first cloned in the vector pDONOR™ 201 (Invitrogen Prod. No.: 11798-014). Subsequently, the coding region of the OK1 protein from the vector obtained was cloned by sequence-specific recombination into the expression vector pDEST™17 (Invitrogen Prod. No.: 11803-014). The expression vector obtained is designated as A.t.-OK1-pDEST™1. The cloning resulted in a translational fusion of the cDNA coding for the A.t-OK1 protein with the nucleotides present in the expression vector pDEST™17 The nucleotides originating from the vector pDEST17™17, which are translationally fused with the cDNA coding the A.t.-OK1 protein, code for 21 amino acids. These 21 amino acids include, amongst others, the start codon (ATG) and a so-called His tag (6 histidine residues directly after one another). After translation of these translationally fused sequences, this

(Invitrogen Prod. No.: A3600). The plasmid obtained is designated A.t.-OK1-pGEM®-T, the cDNA sequence coding for the A.t.-OK1 protein was determined and is shown under SEQ ID NO. 1.®

The sequence shown under SEQ ID NO 1 is not the same as the sequence, which is contained in the database. This has already been discussed for the amino acid sequence coding for an A.t.-OK1 protein.

Conditions used for the amplification of the cDNA coding for the A.t.-OK1 protein

First strand synthesis:

The conditions and buffer specified by the manufacturer were used. In addition, the reaction preparation for the first strand synthesis contained the following substances:

3 µg	Total RNA
5 µM	3'-primer (OK1rev1:5´-GACTCAACCACATAACACACAAAGATC)
0.83 µM	dNTP Mix

The reaction preparation was incubated for 5 minutes at 75°C and subsequently cooled to room temperature.

The 1st strand buffer, RNase inhibitor and DTT were then added and incubated for 2 minutes at 42°C before 1 µL Superscript RT DNA polymerase was added and the reaction preparation incubated for 50 minutes at 42°C.

Conditions for the amplification of the first strand by means of PCR:

1 µL	of the reaction preparation of the first strand synthesis
0.25 µM	3'Primer (OK1rev2:5´- TGGTAACGAGGCAAATGCAGA)
0.25 µM	5'Primer (OK1fwd2:5´-ATCTCTTATCACACCACCTCCAATG)

Reaction conditions:

Step 1	95°C	2 min

Step 2	94°C	20 sec
Step 3	62°C	30 sec
Step 4	68°C	4 minutes
Step 5	94°C	20 sec
Step 6	56°C	30 sec
Step 7	68°C	4 minutes
Step 8	68°C	10 minutes

The reaction was first carried out in accordance with Steps 1 to 4. Ten repeats (cycles) were carried out between Step 4 and Step 2, the temperature of Step 3 being reduced by 0.67°C after each cycle. This was subsequently followed by the reaction in accordance with the conditions specified in Steps 5 to 8. Twenty five repeats (cycles) were carried out between Step 7 and Step 5, the time of Step 7 being increased by 5 sec on each cycle. On completion of the reaction, the reaction was cooled to 4°C.

3. Manufacture of a vector for the recombinant expression of cDNA of the OK1 protein

Following amplification by means of PCR by using the plasmid A.t.-OK1-pGEM® as a template using Gateway Technology (Invitrogen), the sequence coding the OK1 protein from *Arabidopsis thaliana* was first cloned in the vector pDONOR™ 201 (Invitrogen Prod. No.: 11798-014). Subsequently, the coding region of the OK1 protein from the vector obtained was cloned by sequence-specific recombination into the expression vector pDEST™17 (Invitrogen Prod. No.: 11803-014). The expression vector obtained is designated as A.t.-OK1-pDEST™1. The cloning resulted in a translational fusion of the cDNA coding for the A.t-OK1 protein with the nucleotides present in the expression vector pDEST™17 The nucleotides originating from the vector pDEST17™17, which are translationally fused with the cDNA coding the A.t.-OK1 protein, code for 21 amino acids. These 21 amino acids include, amongst others, the start codon (ATG) and a so-called His tag (6 histidine residues directly after one another). After translation of these translationally fused sequences, this

results in an A.t.-OK1 protein, which has the additional 21 amino acids coded for by nucleotides originating from the vector at its N-terminus. The recombinant A.t.-OK1 protein resulting from this vector therefore contains 21 additional amino acids originating from the vector pDEST™17 at its N-terminus.

4. Heterologous expression of the OK1 protein in E. coli

The expression vector A.t.-OK1-pDEST™17 obtained in accordance with Example 3 was transformed in the *E. coli* strain BL21 Star™ (DE3) (Invitrogen, Prod. No. C6010-03). A description of this expression system has already been given above (see Item 3, General Methods). Bacteria clones, containing the vector A.t.-OK1-pDEST™17, resulting from the transformation were first used to manufacture a preliminary culture, which was subsequently used for inoculating a main culture (see Item 3.c, General methods). The preliminary culture and the main culture were each incubated at 30°C under agitation (250 rpm). When the main culture had reached an OD_{600} of ca. 0.8, the expression of the recombinant A.t.-OK1 protein was induced by the addition of IPTG (isopropyl-beta-D-thiogalactopyranoside) until a final concentration of 1 mM was achieved. After the addition of IPTG, the main culture was incubated at 30°C under agitation (250 rpm) until an OD_{600} of ca. 1.8 was achieved. The main culture was then cooled for 30 minutes on ice before the cells of the main culture were separated from the culture medium by centrifugation (10 minutes at 4,000xg and 4°C).

5. Purification of the recombinantly expressed OK1 protein

The purification and concentration of the A.t.-OK1 protein from cells obtained in accordance with Example 4 was carried out using the method described under Item 4, General Methods.

6. Demonstration of starch-phosphorylating activity of the OK1 protein

The starch-phosphorylating activity of the A.t.-OK1 protein was demonstrated in accordance with the method described under Item 11, General Methods. In doing so, 5 µg of purified A.t.-OK1 protein manufactured in accordance with Example 5 was in each case incubated in a Preparation A with 5 mg of starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana* in accordance with Example 1 b) and in a Preparation B with 5 mg of starch obtained by enzymatic phosphorylation in accordance with Example 1 c), in each case in 500 µl of phosphorylation buffer containing 0.05 mM radioactively (^{33}P) labeled, randomised ATP (in total 1,130,00 cpm, ca. 0.55 µCi) for 30 minutes at room temperature under agitation. A Preparation C which corresponded to the Preparation B but contained no OK1 protein but was otherwise treated in the same manner as Preparations A and B was used as control. For all the preparations (A, B, C) two tests were carried out independently of one another in each case.

Using a scintillation counter, the starches from Preparations A, B, and C were investigated for the presence of radioactively labeled phosphate (see Item 11 b), General Methods). The results are shown in Table 1 and in Fig. 3.

	Measured radioactivity [cpm]	
	Test 1	Test 2
Preparation A (non-phosphorylated starch + OK1)	42	47
Preparation B (phosphorylated starch + OK1)	7921	8226
Preparation C (phosphorylated starch without protein)	56	53

Table 1: Demonstration of starch-phosphorylating activity of the OK1 protein

From the results obtained, it can be seen that the OK1 protein does not transfer phosphate groups from ATP to starch when non-phosphorylated starch is provided

as a substrate, as the quota of phosphate groups transferred to non-phosphorylated starch by means of an OK1 protein, measured in cpm, does not exceed the quota of radioactively labeled phosphate groups in Preparation C (control). If, on the other hand, P-starch is provided as a substrate, the quota of radioactive phosphate groups, measured in cpm, which are transferred from ATP to P-starch, is significantly higher. From this, it can be seen that the OK1 protein requires P-starch as a substrate and that non-phosphorylated starch is not accepted as a substrate by the OK1 protein.

If the test described above is carried out with ATP specifically labeled in the gamma position with ^{33}P, then it is not possible to establish any incorporation of radioactively labeled phosphate in the starch. From this, it can be seen that the beta phosphate residue of ATP is transferred from an OK1 protein to starch. The results of such a test are shown in Fig. 6.

7. Demonstration of autophosphorylation

Autophosphorylation of the A.t.-OK1 protein was demonstrated by means of the methods described above (see Item 12, General Methods). Here, 50 µg of purified A.t.-OK1 protein were incubated with radioactively labeled, randomised ATP in 220 µl of phosphorylation buffer (see above, Item 12 d), General Methods) at room temperature for 60 minutes under agitation. Subsequently, 100 µl in each case was removed from the incubation preparations and transferred to four fresh reaction vessels. In reaction vessel 1, the reaction was stopped by the addition of 40 µl 0.11M EDTA. Reaction vessel 2 was incubated at 95°C for 5 minutes. HCl was added to reaction vessel 3 up to a final concentration of 0.5 M, and NaOH was added to reaction vessel 4 up to a final concentration of 0.5 M. Reaction vessels 3 and 4 were each incubated for 25 minutes at 30°C. Subsequently, 50 µl in each case was removed from reaction vessels 1, 2, 3 and 4, mixed with SDS test buffer and separated by means of SDS acrylamide gel electrophoresis (7.5% acrylamide gel). For this purpose, samples from the reaction vessels were applied to each of two identical acrylamide gels. One of the gels obtained on completion of electrophoresis

was subjected to autoradiography, while the second gel was stained with Coomassie Blue.

In the gel stained with Coomassie Blue (see Fig. 2A)), it can be clearly seen that treatment with 0.5 M NaOH leads to degradation of the OK1 protein. The OK1 protein must therefore be described as unstable towards NaOH. Incubations at 30°C, 95°C and with 0.5 M HCl show that the OK1 protein is relatively stable under the stated incubation conditions. This can be concluded from the fact that, under these incubation conditions, in each case approximately the same amounts of OK1 protein can be demonstrated in the gel concerned after staining with Coomassie Blue.

In the autoradiography (see Fig. 2B)), it can be seen by comparison with the phosphorylated OK1 protein incubated at 30°C that an incubation of the phosphorylated OK1 protein at 95°C leads to a significant reduction in the phosphate, which has bound to the OK1 protein. The binding between the phosphate residue and an amino acid of the OK1 protein must therefore be described as heat-unstable. Furthermore, a slight reduction of the phosphate bound to the OK1 protein can also be seen for the incubation with 0.5 M HCl and 0.5 M NaOH in comparison with phosphorylated OK1 protein incubated at 30°C. If the fact is taken into account that the quantity of OK1 protein in the autoradiography after treatment with 0.5 M NaOH is significantly less than in the samples treated with heat and acid on account of the instability of the OK1 protein towards NaOH, then it can be concluded that the binding between the phosphate residue and an amino acid of the OK1 protein will be relatively stable with respect to bases. As the sample treated with acid contains approximately the same amounts of protein as the samples incubated at 30°C and at 95°C, and yet has a significantly lower signal in the autoradiography than the sample treated at 30°C, it must be assumed that acid incubation conditions also split the bond between a phosphate residue and an amino acid of the OK1 protein to a certain extent. An instability of the binding between a phosphate residue and an amino acid of the OK1 protein could therefore also be established in the tests carried out. At the

same time, the instability with respect to acids is significantly less labeled than the instability with respect to heat.

The binding between the amino acid histidine and phosphate are heat-unstable, acid-unstable but base-stable (Rosenberg, 1996, Protein Analysis and Purification, Birkhäuser, Boston, 242-244). The results described above are therefore an indication that a phosphohistidine is produced by the autophosphorylation of an OK1 protein.

If recombinantly expressed OK1 protein is incubated as described above with ATP specifically labeled with ^{33}P in the gamma position, no autophosphorylation can be established. Fig. 5 A) shows the amount of protein which can be detected in the respective reaction preparation by means of Western Blot analysis after the relevant incubation steps. Fig. 5 B) shows an autoradiography of protein from the individual reaction preparations. It can be seen that, when ATP specifically labeled in the gamma position is used, no autophosphorylation of the OK1 protein can be demonstrated, whereas, when randomised ATP is used, autophosphorylation can be demonstrated. This means that when an OK1 protein is autophosphorylated, the phosphate residue of the beta position of the ATP is covalently bound to an amino acid of the OK1 protein.

8. Demonstration of the C-atom positions, which are phosphorylated by an OK1 protein, of the glucose molecules of starch

a) Manufacture of phosphorylated starch

Phosphorylated starch was manufactured in accordance with Item 7, General Methods. To do this, 5 mg of non-phosphorylated starch, isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana* was used in a Preparation A with 25 µg of purified A.t.-OK1 protein and, in a second Preparation B, 5 mg of *in vitro* phosphorylated starch originally isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana* was used with 5 µg of purified R1 protein. The reaction was

carried out in 500 µl of phosphorylation buffer in each case, which, in each case contained ^{33}P labeled ATP (ca. 2.5 x 10^6 cpm), by incubating at room temperature for 1 hour under agitation. In addition, a control preparation was used, which contained 5 mg of starch isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana* and the said phosphorylation buffer, but no protein. The control preparation was treated in exactly the same way as preparations A and B. The individual reactions were stopped by adding 125 µl of 10% SDS in each case and washing was carried out once with 2% SDS, five times with 2 mM ATP and twice with H_2O, using 900 µl in each case. Centrifugation was carried out after each washing step (2 minutes in an Eppendorf table centrifuge at 13,000 rpm in each case). The starch pellets obtained were resuspended 1 ml H_2O in each case and 100 µl of each preparation was mixed after the addition of 3 ml of scintillation cocktail (Ready Safe™, BECKMANN) and subsequently measured using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER™).

The measurement gave the following results:

Control:	63 cpm/100 µL	630 cpm/1000 µl
Preparation A (OK1):	1351 cpm/100 µl	13512 cpm/1000 µl
Preparation B (R1):	3853 cpm/100 µl	38526 cpm/1000 µl

b) Total hydrolysis of the P-starch

The suspensions of Preparations A, B and C obtained in accordance with Step a) were centrifuged again (5 minutes in an Eppendorf table centrifuge at 13,000 rpm), the pellets obtained resuspended in 90 µl 0.7 M HCl (Baker, for analysis) and subsequently incubated for 2 hours at 95°C. Preparations A, B and C were then centrifuged again (5 minutes in an Eppendorf table centrifuge at 13,000 rpm), and the supernatant transferred to a new reaction vessel. Sedimented residues of the preparations were resuspended in 100 ml H_2O in each case and after the addition of 3 ml of scintillation cocktail (Ready Safe™, BECKMANN) were measured using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN

COULTER™). Significant amounts of radioactivity could not be demonstrated in any of the residues, which means that all the hydrolysis products labeled with radioactive phosphate are located in the supernatant.

This was followed by neutralisation of the individual supernatants containing the hydrolysis products by the addition in each case of 30 µl 2 M NaOH (the amount of NaOH required for neutralisation was tested out in advance on blank samples). The neutralised hydrolysis products were placed on a 10 kDa Microcon filter, which had previously been rinsed twice with 200 µl H_2O in each case, and centrifuged for ca. 25 minutes at 12,000 rpm in an Eppendorf table centrifuge. 10 µl was taken from the filtrate obtained (ca. 120 µl in each case) and, after the addition of 3 ml of scintillation cocktail (Ready SafeTM, BECKMANN), were measured using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTERTM). The determination of the activity present in the individual preparations gave the following results:

Preparation A (OK1): 934 cpm/10 µl 11,208 cpm/120 µl 93 cpm/µl

Preparation B (R1): 2518 cpm/10 µl 30,216 cpm/120 µl 252 cpm/µl

c) Separation of the hydrolysis products

The hydrolysis products obtained in accordance with Step b) were separated by means of HPAE using a Dionex system under the conditions stated above (see General Methods, Item 13 c)). The samples for separating the filtered supernatants of Preparations A and B obtained in accordance with Step b) were composed as follows:

Preparation A (OK1): 43 µl of the supernatant of Preparation A obtained in accordance with Step b) (equivalent to ca. 4,000 cpm), 32 µl H_2O, 2.5 µl 2.5 mM glucose-6-phosphate and 2.5 µl 5 mM glucose-3-phosphate (Σ Volume = 80 µl).

Preparation B (R1): 16 µl of the supernatant of Preparation B obtained in accordance with Step b) (equivalent to ca. 4,000 cpm), 59 µl H_2O, 2.5 µl 2.5 mM glucose-6-phosphate and 2.5 µl 5 mM glucose-3-phosphate (Σ Volume = 80 µl).

In each case 60 µl, containing ca. 3,000 cpm, of the corresponding samples was injected for separation using HPAE. The HPAE was carried out in accordance with the conditions specified under Point 23 c). After passing through the HPAE column, the elution buffer was collected in fractions, each of 1 ml. Collection of the fractions was begun 10 minutes after injecting the sample. Based on the signal received from the PAD detector used, the elution of glucose-6-phosphate was assigned to fraction 15 and the elution of glucose-3-phosphate to fraction 17. In each case, 500 µl of the individual fractions were mixed with 3 ml of scintillation cocktail (Ready SafeTM, BECKMANN) and subsequently measured using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTERTM). The following measurements were obtained for the individual fractions:

	Total cpm per fraction	
	Preparation A (OK1)	Preparation B (R1)
Fr 13	8.7	3.3
Fr 14	13.1	32.2
Fr 15 (G6P)	207.3	1952.8
Fr 16	3998	112.3
Fr 17 (G3P)	1749.2	801.6
Fr 18	196.7	17.3
Fr 19	6.7	18.9
Total	2581.5	2938.3
Deposit	3000.0	3000.0

Recovery	86.0%	97.9%

Table 4: Measured amounts of radioactivity [cpm] in individual fractions of hydrolysis products obtained by hydrolysis of starch phosphorylated by means of an OK1 protein or R1 protein.

The results are also shown graphically in Fig. 5.

After phosphorylation of starch catalysed by R1 protein, ca. 66% of the radioactively labeled phosphate, referred to the total measured radioactive phosphate in the analysed fractions, eluted after hydrolysing the starch with the fraction which contained glucose-6-phosphate as standard, and ca. 27% with the fraction which contained glucose-3-phosphate as standard. After phosphorylation of starch catalysed by OK1 protein, ca. 67% of the radioactively labeled phosphate, referred to the total measured radioactive phosphate in the analysed fractions, eluted after hydrolysing the starch with the fraction which contained glucose-3-phosphate as standard, and ca. 8% with the fraction which contained glucose-6-phosphate as standard. From this, it can be concluded that glucose molecules of the starch of R1 proteins are preferably phosphorylated in the C-6 position, whereas from OK1 proteins glucose molecules of the starch are preferably phosphorylated in the C-3 position.

9. Identification of an OK1 protein in rice

Using the methods described under Items 1 to 13, General Methods, it was also possible to identify a protein from *Oryza sativa* (variety M202), which transfers a phosphate residue from ATP to P-starch. The protein was designated as O.s.-OK1. Non-phosphorylated starch is not used by the O.s.-OK1 protein as a substrate, i.e. the O.s.-OK1 protein also does need P-starch as a substrate. The nucleic acid sequence defining the identified O.s.-OK1 protein is shown under SEQ ID NO 3 and the amino acid sequence coding for the O.s.-OK1 protein is shown under SEQ ID NO. 4. The amino acid sequence coding for the O.s.-OK1 protein shown under SEQ ID NO 4 has an identity of 57% with the amino acid sequence coding for the A.t.-OK1

protein shown under SEQ ID NO 2. The nucleic acid sequence coding for the O.s.-OK1 protein shown under SEQ ID NO 3 has an identity of 61% with the nucleic acid sequence coding for the A.t.-OK1 protein shown under SEQ ID NO 1.

Manufacture of the plasmid pMI50 containing the nucleic acid sequence coding for an OK1 protein from *Oryza sativa*

The vector pMI50 contains a DNA fragment, which codes for the complete OK1 protein from rice of the variety M202.

The amplification of the DNA from rice was carried out in five sub-steps.

The part of the open reading frame from position -11 to position 288 of the sequence specified under SEQ DIE NO 3 was amplified using reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-R9 (GGAACCGATAATGCCTACATGCTC) and Os_ok1-F6 (AAAACTCGAGGAGGATCAATGACGTCGCTGCGGCCCCTC) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML123.

The part of the open reading frame from position 250 to position 949 of the sequence specified under SEQ DIE NO 3 was amplified using reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F4 (CCAGGTTAAGTTTGGTGAGCA) and Os_ok1-R6 (CAAAGCACGATATCTGACCTGT) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML120.

The part of the open reading frame from position 839 to position 1761 of the sequence specified under SEQ DIE NO 3 was amplified using reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F7 (TTGTTCGCGGGATATTGTCAGA) and Os_ok1-R7 (GACAAGGGCATCAAGAGTAGTATC)

as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML121.

The part of the open reading frame from position 1571 to position 3241 of the sequence specified under SEQ DIE NO 3 was amplified using reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F8 (ATGATGCGCCTGATAATGCT) and Os_ok1-R4 (GGCAAACAGTATGAAGCACGA) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML119.

The part of the open reading frame from position 2777 to position 3621 was amplified using polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F3 (CATTTGGATCAATGGAGGATG) and Os_ok1-R2 (CTATGGCTGTGGCCTGCTTTGCA) as a primer on genomic DNA of rice. The amplified DNA fragment was cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML122.

The cloning together of the sub-parts of the open reading frame of OK1 was carried out as follows.

A 700 base pair long *Apa*I fragment of pML120, containing part of the open reading frame of OK1, was cloned in the *Apa*I site of pML121. The plasmid obtained was designated as pMl47.

A 960 base pair long fragment containing the regions of the vectors from pML120 and pML123 coding for OK1 was amplified by means of polymerase chain reaction. In doing so, the primers Os_ok1-F4 (see above) and Os_ok1-R9 (see above), each in a concentration of 50 nm, and the primers Os_ok1-F6 and Os_ok1-R6, each in a concentration of 500 nm, were used. The amplified DNA fragment was cloned into

the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pMI44.

An 845 base pair long fragment of pML122 was reamplified for introducing a *Xho*I site after the stop codon with the primers Os_ok1-F3 (see above) and Os_ok1-R2Xho (AAAACTCGAGCTATGGCTGTGGCCTGCTTTGCA) and cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as t pMI45.

A 1671 base pair long fragment containing part of the open reading frame of OK1 was obtained from pML119 by digesting with the restriction enzymes *Spe*I and *Pst*I. The fragment was cloned into pBluescript II SK+ (Genbank Acc.: X52328). The plasmid obtained was designated as pMI46.

A 1706 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Spe*I and *Xho*I from pMI46 and cloned into the vector pMI45, which had been excised with the same restriction enzymes. The plasmid obtained was designated as pMI47.

A 146 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Afl*II/*Not*I from pMI43 and cloned into the vector pMI44, which had been excised with the same restriction enzymes. The plasmid obtained was designated as pMI49.

A 1657 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Not*I and *Nar*I from the vector pMI49 and cloned into the vector pMI47, which had been excised with the same restriction enzymes. The plasmid obtained was designated as pMI50 and contains the whole coding region of the OK1 protein identified in rice.

10. Identification of further OK1 proteins from various plant species

Using the methods described under Items 1 to 13, General Methods, proteins which transfer a phosphate residue from ATP to P-starch were also identified in barley (*Hordeum vulgare*), potato (*Solanum tuberosum*), wheat (*Triticum aestivum*) and millet (*Sorghum bicolor*). Non-phosphorylated starch is not used as substrate by these proteins, i.e., these proteins require P-starch as substrate.

The proteins were isolated using the method described under Item 14, General Methods, digested with trypsin, dissolved out of the gel and sequenced using Q-TOF-MS-MS. Using the peptide sequences obtained, it was possible to determine EST nucleic acid sequences which code for the relevant OK1 proteins from barley, potato, wheat or millet by means of database comparisons (blast searches).

The nucleic acid sequence shown in SEQ ID NO 9 codes for a part of an OK1 protein from barley and was traced under "Accession" No.: TC117610 in the TIGR (http://tigrblast.tigr.org/tgi/) database by means of a database comparison (blast search). Those peptides which were obtained by sequencing the OK1 protein isolated from barley using Q-TOF-MS-MS and were used to identify the EST nucleic acid sequence shown under SEQ ID NO 9, are specified in SEQ ID NO 6, SEQ ID NO 7 and SEQ ID NO 8. The amino acid sequence shown in SEQ ID NO 10 codes for a part of an OK1 protein from barley and can be derived from the nucleic acid sequence shown in SEQ ID NO 10.

The nucleic acid sequence shown in SEQ ID NO 15 codes for a part of an OK1 protein from potato and was found under "Accession" No.: BF054632 in the TIGR (http://tigrblast.tigr.org/tgi/) database by means of a database comparison (blast search). Those peptides which were obtained by sequencing the OK1 protein isolated from potato using Q-TOF-MS-MS and were used to identify the EST nucleic acid sequence shown under SEQ ID NO 15, are specified in SEQ ID NO 11, SEQ ID

NO 12, SEQ ID NO 13 and SEQ ID NO 14. The amino acid sequence shown in SEQ ID NO 16 codes for a part of an OK1 protein from potato and can be derived from the nucleic acid sequence shown in SEQ ID NO 15.

The nucleic acid sequence shown in SEQ ID NO 21 codes for a part of an OK1 protein from millet and was found under "Accession" No.: TC77219 in the TIGR (http://tigrblast.tigr.org/tgi/) database by means of a database comparison (blast search). Those peptides which were obtained by sequencing the OK1 protein isolated from millet using Q-TOF-MS-MS and were used to identify the EST nucleic acid sequence shown under SEQ ID NO 21, are specified in SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19 and SEQ ID NO 20. The amino acid sequence shown in SEQ ID NO 22 codes for a part of an OK1 protein from millet and can be derived from the nucleic acid sequence shown in SEQ ID NO 21.

The nucleic acid sequence shown in SEQ ID NO 25 codes for a part of an OK1 protein from wheat and was found under "Accession" No.: CA74319 in the TIGR (http://tigrblast.tigr.org/tgi/) database by means of a database comparison (blast search). Those peptides which were obtained by sequencing the OK1 protein isolated from wheat using Q-TOF-MS-MS and were used to identify the EST nucleic acid sequence shown under SEQ ID NO 25, are specified in SEQ ID NO 23 and SEQ ID NO 24. The amino acid sequence shown in SEQ ID NO 26 codes for a part of an OK1 protein from wheat and can be derived from the nucleic acid sequence shown in SEQ ID NO 25.

The following settings were selected to carry out the database comparisons:

Program: tblastn

Matrix: blosum62

Expect: 100

Echofilter: disabled

Descriptions: 20

All other settings read "default".

11. Manufacture of an antibody, which specifically recognises an OK1 protein

As an antigen, ca. 100 µg of purified A.t.-OK1 protein was separated by means of SDS gel electrophoresis, the protein bands containing the A.t.-OK1 protein excised and sent to the company EUROGENTEC S.A. (Belgium), which carried out the manufacture of the antibody under contract. First, the preimmune sera of rabbits were investigated to see whether they would already recognise a protein from an A. t. total extract before immunisation with recombinant OK1. The preimmune sera of two rabbits recognised no proteins in the range 100-150 kDa and were thus chosen for immunisation. Four injections of 100 µg of protein (day 0, 14, 28, 56) were given to each rabbit. Four blood samples were taken from each rabbit: (day 38, day 66, day 87 and the final bleeding). Serum obtained after the first bleeding, already showed a specific reaction with OK1 antigen in Western blot. However, in all further tests, the final bleeding of a rabbit was used

12. Manufacture of transgenic rice plants which have an elevated or a reduced activity of an OK1 protein

a) Manufacture of the plasmid pGlo-A.t.-OK1

The plasmid pIR94 was obtained by amplifying the promoter of the globulin gene from rice by means of a polymerase chain reaction (30 x 20 sec 94 °C, 20 sec 62 °C, 1 min 68 °C, 4 mM Mg2SO4) with the primers glb1-F2 (AAAACAATTGGCGCCTGGAGGGAGGAGA) and glb1-R1 (AAAACAATTGATGATCAATCAGACAATCACTAGAA) on the genomic DNA of rice of the variety M202 with High Fidelity Taq Polymerase (Invitrogen, catalogue number 11304-011) and cloned into pCR2.1 (Invitrogen catalogue number K2020-20).

The plasmid pIR115 was obtained by cloning a synthetic piece of DNA consisting of the two oligonucleotides X1 (TGCAGGCTGCAGAGCTCCTAGGCTCGAGTTAACACTAGTAAGCTTAATTAAGAT ATCATTTAC) and X2

(AATTGTAAATGATATCTTAATTAAGCTTACTAGTGTTAACTCGAGCCTAGGAGCT CTGCAGCCTGCA) into the vector pGSV71 excised with *Sda*I and *Mun*I.

The plasmid pIR115 obtained was excised with *Sda*I, the protruding 3'-ends smoothed with T4 DNA polymerase and a 197-base-pair *HindIII / SphI* fragment from pBinAR (Höfgen and Willmitzer, 1990, Plant Science 66, 221-230), smoothed by means of T4 DNA polymerase and containing the termination signal of the octopine synthase gene from *Agrobacterium tumefaciens*, was inserted. The plasmid obtained was designated as pIR96.

The plasmid pIR103 was obtained by cloning a 986-base-pair long DNA fragment from pIR94, containing the promoter of the globulin gene from rice, into the plasmid pIR96.

pGSV71 is a derivative of the plasmid pGSV7, which is derived from the intermediary vector pGSV1. pGSV1 is a derivative of pGSC1700 whose construction has been described by Cornelissen and Vanderwiele (Nucleic Acid Research 17, (1989), 19-25). pGSV1 was obtained from pGSC1700 by deletion of the carbenicillin resistance gene, as well as deletion of the T-DNA sequences of the TL-DNA region of the plasmid pTiB6S3.

pGSV7 contains the replication origin of the plasmid pBR322 (Bolivar et al., Gene 2, (1977), 95-113) as well as the replication origin of the *Pseudomonas* plasmid pVS1 (Itoh et al., Plasmid 11, (1984), 206). pGSV7 also contains the selectable marker gene *aadA*, from the transposon Tn1331 from *Klebsiella pneumoniae*, which imparts resistance to the antibiotics spectinomycin and streptomycin (Tolmasky, Plasmid 24 (3), (1990), 218-226; Tolmasky and Crosa, Plasmid 29(1), (1993), 31-40)

The plasmid pGSV71 was obtained by cloning a chimeric *bar* gene between the border regions of pGSV7. The chimeric *bar* gene contains the promoter sequence of the cauliflower mosaic virus for initiation of the transcription (Odell et al., Nature 313, (1985), 180), the *bar* gene from *Streptomyces hygroscopicus* (Thompson et al.,

Embo J. 6, (1987), 2519-2523) and the 3'-untranslated region of the nopaline synthase gene of the T-DNA of pTiT37 for termination of the transcription and polyadenylation. The *bar* gene provides tolerance against the herbicide glufosinate ammonium.

A DNA fragment which contains the complete open reading frame of the OK1 protein from *Arabidopsis* was excised from the vector A.t.-ok1-pGEM-T and cloned into the vector pIR103. For this purpose the plasmid A.t.-OK1-pGEM-T was excised with the restriction enzyme *Bsp*120I, the ends smoothed with T4-DNA polymerase and subsequently excised with *Sal*I. The DNA fragment coding for the OK1 protein from *Arabidopsis thaliana* was cloned into the vector pIR103 excised with *Ecl*136II and *Xho*I. The plasmid obtained was designated as pGlo-A.t.-OK1.

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b) Manufacture of a construct for inhibiting the OK1 protein in rice by means of RNAi technology

The plasmid pML125, which was used for the transformation of rice plants, was obtained by specific recombination of the plasmids pML124 and pIR115 using the Gateway™ cloning system (Invitrogen).

pML124 was obtained by cloning a 359 base pair long DNA fragment of pML119 (see above, Example 9), containing part of the open reading frame which codes for the OK1 protein from rice, into the vector pENTR-1A (Invitrogen, product number 11813-011) excised with *Eco*RI.

The plasmid pIR87 was obtained by amplifying the intron 1 of the gene coding for alcohol hydrogenase from maize with the primers Adh(i)-1 (TTTTCTCGAGGTCCGCCTTGTTTCTCCT) and Adh(i)-2 (TTTTCTCGAGCTGCACGGGTCCAGGA) on the genomic DNA of maize. The product of the polymerase chain reaction (30 x 30 sec 94 °C, 30 sec 59 °C, 1 min 72 °C, 2.5 mM $MgCl_2$) was digested with the restriction enzyme *Xho*I and cloned into the vector

pBluescript II SK+ (Genbank Acc.: X52328), which had been excised with the same enzyme.

A 986 base pair long DNA fragment from pIR94, containing the promoter of the globulin gene from rice, was cloned into the vector pIR96. The plasmid obtained was designated as pIR103.

The plasmid pIR107 was obtained by cloning the "RfA cassette" (see above) into the plasmid pIR103 excised with the restriction enzyme *Eco*RV.

A 540 base pair long fragment containing the intron 1 of the gene coding for alcohol dehydrogenase from maize was excised from the plasmid pIR87 with the restriction enzyme *Xho*I and cloned into the plasmid pIR107 likewise excised with *Xho*I. The plasmid obtained was designated as pIR114. The plasmid pIR115 was obtained by cloning the "RfA cassette" (see above) into the plasmid pIR114 excised with *Ecl*136II.

c) Transformation of rice plants

Rice plants (variety M202) were transformed using *Agrobacterium* (containing either the plasmid pGlo-A.t.-OK1 or the plasmid pML125) using the method described in Hiei et al. (1994, Plant Journal 6(2), 271-282).

d) Analysis of the transgenic rice plants which expressed the A.t.-OK1 protein and the starch synthesised by these plants

Plants transformed with the plasmid pGlo-A.t.-OK1 which exhibited an expression of the heterologous A.t.-OK1 protein were identified by means of a Northern Blot analysis.

Plants which exhibited a detectable quantity of mRNA coding for A.t.-OK1 protein were cultivated in the greenhouse. Grains of these plants were harvested. Starch from these grains showed an elevated content of phosphate covalently bound to the starch concerned.

e) Analysis of the transgenic rice plants in which the expression of the endogenous OK1 protein was repressed by means of RNAi technology and the starch synthesised from these plants

Rice plants which were transformed with the plasmid pML125 and exhibited a reduced expression of the endogenous mRNA coding for the OK1 protein were identified by means of Northern Blot analysis.

13. Manufacture of transgenic potato plants which have an elevated or a reduced activity of an OK1 protein

a) Manufacture of the plasmid pBinB33-Hyg

Starting from the plasmid pBinB33, the *Eco*RI-*Hind*III fragment including the B33 promoter, a part of the polylinker, and the *ocs* terminator were excised and ligated into the correspondingly excised vector pBIB-Hyg (Becker, 1990, Nucl. Acids Res. 18, 203). Acids Res. 18, 203).

The plasmid pBinB33 was obtained by ligating the promoter of the patatin gene B33 from *Solanum tuberosum* (Rocha-Sosa et al., 1989) as a *Dra*I fragment (nucleotide –1512 - +14) into the vector pUC19 excised with *Sst*I, the ends of which had been smoothed using the T4 DNA polymerase. This resulted in the plasmid pUC19-B33. The B33 promoter was excised from this plasmid with *Eco*RI and *Sma*I and ligated into the correspondingly excised vector pBinAR (Höfgen and Willmitzer, 1990, Plant Science 66, 221-230). This resulted in the plant expression vector pBinB33.

b) Manufacture of the vector A.t.-OK1-pBinB33-Hyg

The coding sequence of the A.t.-OK1 protein was excised with the restriction endonucleases *Bsp120I* and *SalI* from the plasmid OK1-pGEM and ligated into the vector pBinB33-Hyg excised with *SmaI* and *SalI*. The plasmid obtained was designated as A.t.-OK1-pBinB33-Hyg.

c) Transformation of potato plants

Agrobacterium tumefaciens (strain GV2260) was transformed with the plasmid A.t.-OK1-pBinB33-Hyg. Potato plants of the Désirée variety were then transformed using agrobacteria containing the plasmid A.t.-OK1-pBinB33-Hyg using the method described in Rocha-Sosa et al. (EMBO J. 8, (1989), 23-29) and plants regenerated. The plants obtained from this transformation event were designated 385JH.

d) Analysis of the transgenic potato plants and the starch synthesised by these

Plants which exhibited an elevated activity of the heterologously expressed A.t.-OK1 protein and also plants in which the activity of the endogenous OK1 protein was reduced by a co-suppression effect were identified by means of a Western Blot analysis. The Western Blot analysis was carried out using the antibody described under Example 11

Fig. 7 exemplary shows the detection of the A.t.-OK1 Protein in single plants from the transformation event 385JH by means of Western Blot analysis. For induction of the B33 Promotor in leaf tissue single lines of the the transformation event 385JH were cultivated on solidified Murashige Skoog medium containing 100 mM sucrose in tissue culture for two days. After harvest protein extracts were produced from leaf tissue of these plants according to the method described under General Methods, Item 1a).. After separation of the proteins by means of denaturing polyacrylamide gel electrophoreses 40 µg protein extract of each line was analysed by means of Western Blot analysis using the antibody described under Examples, Item 10. As

control samples, protein extracts from Arabidopsis plants and from potato wildtype plants (cv Désirée) were also analysed.

Plants which exhibited an elevated quantity of A.t.-OK1 protein compared to the corresponding wild type plants were cultivated in the greenhouse. Starch which was isolated from tubers of these plants showed an elevated content of phosphate covalently bound to the starch compared to the starch isolated from non-transformed wild type plants.

14. Analysis of Arabidopsis thalliana plants which exhibit a reduced activity of a protein according to the invention

T-DNA insertion mutants of *Arabidopsis thaliana* (available from the Salk Institute Genomic Analysis Laboratory, 10010 N. Torrey Pines Road, La Jolla, CA 92037, http://signal.salk.edu/ under ACC. No.: Salk_110814, Alias N610814), which were homozygotic with respect to insertion in the OK1 gene, were grown under the following conditions:

Light phase: 16 hours, 20°C

Dark phase: 8 hours, 16°C

Shortly before the flowers developed, the plants were cultivated in a light phase of 12 hours at 20°C and a dark phase of 12 hours at 17°C.

Plants of the mutant line obtained (Salk_110814) were cultivated from 3 different seeds of the original seed material (Salk_110814-1, Salk_110814-2, Salk_110814-3) for analysis.

At the end of the dark phase, 10 leaves were removed in each case from 6 wild type plants (Ökotyp Columbia) and decolourised in 70% ethanol at 50°C. Furthermore, 6 leaves were removed in each case from respectively 4 different plants of the mutant

lines Salk_110814-1, Salk_110814-2 or Salk_110814-3 which were in each case homozygotic with respect to T-DNA insertion in an OK1 gene, and these were decolourised in 70% ethanol at 50°C. The leaves were then incubated for 10 minutes in Lugol's solution before excess Lugol's solution was rinsed off the leaves with tap water. All leaves from wild type plants showed no staining with Lugol's solution. On the other hand, all leaves of the mutant lines Salk_110814-1, Salk_110814-2 or Salk_110814-3 showed a dark brown or black colouration (see Fig. 7). The mutant lines therefore showed a starch excess phenotype compared to the wild type plants. During cultivation no differences relating to the growth could be established between the mutant lines and the wild type plants.

Genetically modified *Arabidopsis thaliana* plants which were transformed with an RNAi construct containing "inverted repeats" of the coding region of an OK1 gene under control of the 35S promoter, were analysed with the aid of Western blot analysis using the antibody described in Example 10. Several independent lines which exhibited a reduced quantity of OK1 protein compared to wild type plants were identified. These lines were cultivated under the culture conditions specified above. In each case, 5 leaves of the individual lines were removed at the end of the dark phase (12 hours at 17°C), decolourised in ethanol and stained with Lugol's solution. All the plants showed a starch excess phenotype compared to corresponding wild type plants. During cultivation no differences relating to growth could be established between the genetically modified plants and the wild type plants. The plants genetically modified by means of RNAi technology thus showed the same properties as the mutant lines Salk_110814-1, Salk_110814-2 or Salk_110814-3.

In each case four *Arabidopsis thaliana* plants of the lines A.t.-alpha-OK1-1, A.t.-alpha-OK1-2, A.t.-alpha-OK1-3, A.t.-alpha-OK1-4, A.t.-alpha-OK1-5, resulting from independent transformation events, in which the quantity of OK1 protein is reduced by means of RNAi technology, were investigated for their starch content at different times. The reduction in the quantity of OK1 protein in the respective lines was demonstrated by means of Western blot analysis (see Fig. 8). The leaf starch content of the individual lines was determined using the starch kits from Boehringer Mannheim (Product No.: 0207748). For this purpose, in each case all the leaves of

four plants of the individual lines were harvested and the leaves were homogenised using mortars. 40 mg to 60 mg of the homogenised leaf material was washed twice with 80% ethanol in each case and the supernatant was discarded. The remaining material, which is not soluble in ethanol, was freeze-dried after being washed once in 1 ml of water, then dissolved in 0.5 ml of 0.2M KOH at 95°C for 1 h and the solution obtained was adjusted to pH 7 using 88 µL of 1M acetic acid. 25 µl of the respective solution obtained was mixed with 50 µl of amyloglucosidase solution (Starch–Kit from Boehringer Mannheim, Product No.: 0207748), to which 1 unit of alpha-amylase (from Bacillus amyloliquefaciens, Boehringer, Prod-No. 161764) had been added and was incubated for 1 h at 55 °C. 20 µl of the solution treated with amyloglucosidase and alpha-amylase was then used to determine the glucose using an enzymatic coupled photometric test (see product information sheet for the determination of native starch from Boehringer Inmgelheim, Product No.: 0207748) At the same time as the transgenic lines, the starch content was also determined in leaves of *Arabidopsis thaliana* wild type plants (Ecotype Columbia). The wild type plants and the transgenic plants were cultivated under the same conditions: 12 hours light phase followed by 12 hours dark phase.

Leaves of the respective transgenic plant lines and wild type plants were harvested in each case ca. 4.5 weeks after seed germination after the end of the dark phase, after the end of a light phase and after the end of a second dark phase which directly followed the light phase. For each transgenic plant line, two independent extracts were produced in each case, from which two measurements of the starch content were made in each case. For wild type plants four extracts were produced in each case from which two measurements of the starch content were made in each case. The determination of the leaf starch contents yielded the following results:

Line	Starch content (mg/g FW)	Standard deviation*

	Line	Starch content (mg/g FW)	Standard deviation*
End dark phase 1			
	A.t.-alpha-OK1-1	4.09	0.55
	A.t.-alpha-OK1-2	4.93	0.94
	A.t.-alpha-OK1-3	5.59	0.52
	A.t.-alpha-OK1-4	6.36	0.87
	A.t.-alpha-OK1-5	1.49	0.99
	Wild type	0.78	0.14
End light phase			
	A.t.-alpha-OK1-1	9.30	0.96
	A.t.-alpha-OK1-2	9.86	1.45
	A.t.-alpha-OK1-3	11.68	1.60
	A.t.-alpha-OK1-4	9.53	1.25
	A.t.-alpha-OK1-5	6.61	0.71
	Wild type	5.61	0.72
End dark phase 2			
	A.t.-alpha-OK1-1	3.92	0.83
	A.t.-alpha-OK1-2	4.35	1.07
	A.t.-alpha-OK1-3	6.00	0.63

Line	Starch content (mg/g FW)	Standard deviation*
A.t.-alpha-OK1-4	5.34	1.35
A.t.-alpha-OK1-5	1.46	0.56
Wild type	0.62	0.18

Table 4: Quantity of leaf starch in *Arabidopsis thaliana* plants in which the quantity of OK1 protein is reduced using RNAi technology.

* Standard deviation using the general formula: root $[(n\Sigma x^2 - (\Sigma x)^2) / n(n-1)]$

15. Analysis of starch isolated from plants which exhibit a reduced activity of an OK1 protein

Starch was isolated from leaves of the plants described in Example 14 and hydrolysed using the method described under General Methods, Item 13 and then separated by means of HPAE analysis. The areas of the separated signals obtained by means of HPAE analysis for C–3 phosphate and C-6 phosphate were calculated (Software: Chromelion 6.20 from Dionex, USA) and the values obtained were given as the ratio to one another. The ratio of C-6 phosphate to C-3 phosphate in wild type plants was 2.1. In the plants described in Example 14 in which the activity of the OK1 protein was reduced by means of RNAi technology, on the other hand, the average ratio of C-6 phosphate to C-3 phosphate determined by analysing the starch isolated from the lines A.t.-alpha-OK1-1, A.t.-alpha-OK1-2, A.t.-alpha-OK1-3, A.t.-alpha-OK1-4 und A.t.-alpha-OK1-5 was 2.5. The analysis of starch from the line A.t.-alpha-OK1-5 yielded the lowest ratio of C-6 phosphate to C-3 phosphate (ratio of 2.2), starch from the line A.t.-alpha-OK1-1 yielded the highest ratio (ratio of 2.7).

Starch isolated from leaves of the mutants described in Example 13 which exhibit a reduced activity of an OK1 protein showed an increase in the ratio of C-6 phosphate to C-3 phosphate in the starch concerned.